

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C07K 14/575, 14/72, A61K 38/22, C07K 16/26, C12N 15/16, G01N 33/74</b>		<b>A1</b>	(11) International Publication Number: <b>WO 98/58962</b> (43) International Publication Date: 30 December 1998 (30.12.98)
(21) International Application Number: <b>PCT/JP98/02765</b> (22) International Filing Date: 22 June 1998 (22.06.98) (30) Priority Data: 9/165437 23 June 1997 (23.06.97) <b>JP</b> (71) Applicant (for all designated States except US): <b>TAKEDA CHEMICAL INDUSTRIES, LTD. [JP/JP]; 1-1, Doshomachi 4-chome, Chuo-ku, Osaka-shi, Osaka 541-0045 (JP).</b> (72) Inventors; and (75) Inventors/Applicants (for US only): <b>HINUMA, Shuji [JP/JP]; 7-9-1402, Kasuga 1-chome, Tsukuba-shi, Ibaraki 305-0821 (JP). KAWAMATA, Yuji [JP/JP]; 22-2-203, Matsushiro 4-chome, Tsukuba-shi, Ibaraki 305-0035 (JP). FUJII, Ryo [JP/JP]; 7-9-303, Kasuga 1-chome, Tsukuba-shi, Ibaraki 305-0821 (JP). MATSUMOTO, Hirokazu [JP/JP]; 7-9-1204, Kasuga 1-chome, Tsukuba-shi, Ibaraki 305-0821 (JP).</b> (74) Agents: <b>ASAHINA, Tadao et al.; Osaka Plant of Takeda Chemical Industries, Ltd., 17-85, Jusohonmachi 2-chome, Yodogawa-ku, Osaka-shi, Osaka 532-0024 (JP).</b>			(81) Designated States: <b>AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, GW, HU, ID, IL, IS, KG, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, SL, TJ, TM, TR, TT, UA, US, UZ, VN, YU, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</b>  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>

(54) Title: **PROLACTIN SECRETION MODULATOR**

## (57) Abstract

The present invention relates to a ligand polypeptide prolactin secretion modulating activity, and has a function of modulating placental function. The ligand polypeptide can be used as a prolactin secretion-stimulating agent for the prevention and treatment of certain diseases associated with prolactin secretion, such as hypoovarianism, gonocyst cacogenesis, menopausal syndrome, and euthyroid hypometabolism. In addition, the ligand polypeptide of the invention can be used with advantage as an aphrodisiac. The ligand polypeptide of the invention can be used with advantage as a prolactin secretion inhibitory agent in the prevention and treatment of certain diseases associated with prolactin secretion, such as pituitary adenomatosis, brain tumor, emmeniopathy, autoimmune disease, prolactinoma, infertility, impotence, amenorrhea, galactorrhea, acromegaly, Chiari-Frommel syndrome, Argonz-del Castillo syndrome, Forbes-Albright syndrome, lymphoma, Sheehan syndrome or dyszoospermia. In addition, the ligand polypeptide of the present invention is used as an agent for treating or preventing choriocarcinoma, hydatid mole, irruption mole, abortion, unthrifty fetus, abnormal saccharometabolism, abnormal lipidmetabolism or oxytocia.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

DESCRIPTION  
PROLACTIN SECRETION MODULATOR

[Technical Field]

5       The present invention relates to an agent for modulating prolactin secretion and/or placental function, comprising a ligand polypeptide for a G protein-coupled receptor protein.

10      [Background Art]

Many hormones and neurotransmitters mediate biological functions through specific receptors present on the cell membrane. Many of these receptors engage themselves in the intracellular transduction of signals through activation of the coupled guanine nucleotide-binding protein (hereinafter sometimes referred to briefly as G protein) and have the common structure comprising 7 transmembrane domains. Therefore, these receptors are collectively referred to as G protein-coupled receptor or 7-transmembrane receptor.

20       One of the pathways to modulate biological functions mediated by such hormones or neurotransmitters through G protein-coupled receptors is the hypothalamo-pituitary system. Thus, the secretion of pituitary hormone from the hypophysis is controlled by hypothalamic hormones (pituitatropic releasing factor) and the functions of the target cells or organs are regulated through the pituitary hormones released into the circulation. This pathway carries out functional modulations of importance to the living body, such as homeostasis and regulation of the reproduction, development, metabolism and growth of individuals. The secretion of pituitary hormones is controlled by a positive or negative feedback mechanism involving hypothalamic hormone and the peripheral hormone secreted from the target endocrine gland. The

25  
30  
35

various receptor proteins present in the hypophysis are playing a central role in the regulation of the hypothalamus-pituitary system.

5       Meanwhile, it is known that these hormones and factors as well as their receptors are not localized in the hypothalamus-pituitary system but are broadly distributed in the brain. Therefore, it is suspected that, in the central nervous system, this substance called hypothalamus hormone is functioning as a  
10       neurotransmitter or a neuromodulator. Moreover, the substance is distributed in peripheral tissues as well and thought to be playing important roles in the respective tissue.

15       The pancreas is playing a crucial role in the carbohydrate metabolism by secreting glucagon and insulin as well as digestive juice. While insulin is secreted from the pancreatic  $\beta$  cells, its secretion is mainly stimulated by glucose. However, it is known that  $\beta$  cells have a variety of receptors and the  
20       secretion of insulin is controlled by a number of factors in addition to glucose as well as peptide hormones, e.g. galanin, somatostatin, gastric inhibitory polypeptide, glucagon, amylin, etc.; sugars, e.g. mannose etc.; amino acids, and neurotransmitters,  
25       among others.

      The means only heretofore available for identifying ligands for said G protein-coupled receptor proteins is estimation from the homology in primary structure of G protein-coupled receptor proteins.

30       Recently, investigation for novel opioid peptides by introducing a cDNA coding for a receptor protein to which a ligand is unknown, i.e. an orphan G protein-coupled receptor protein, into animal cells has been reported (Reinscheid, R. K. et al., Science, 270, 792-  
35       794, 1995, Menular, J.-C., et al., Nature 377, 532-535, 1995). However, in view of similarities to known G



protein-coupled receptor proteins and tissue distributions, it could be easily anticipated in these cases that the ligand would be belonging to the family of opioid peptides. The history of research and development in the realm of substances acting on the living body through the opioid receptor dates back to many years ago and various antagonists and agonists had been developed. Therefore, among the compounds artificially synthesized, an agonist of the receptor was picked out and, using it as a probe, expression of the receptor in the receptor cDNA-transfected cells was verified. Then, a search was made for an activator of the intracellular signal transduction which was similar to the agonist, the activator so found was purified, and the structure of the ligand was determined. However, when the homology of an orphan receptor to known G protein-coupled receptor proteins is low, it was very difficult to predict its ligand.

As Examples of the orphan G protein-coupled receptor, a human receptor protein (Genomics, vol.29, 335 (1995)) which is encoded by phGR3 (sometimes called GPR10) gene and a rat receptor protein, UHR-1 (Biochem. Biophys. Res. Commun., vol/209, 606 (1995)), is known.

Ligands for orphan G protein-coupled receptors expressed in the hypophysis, central nervous system, and pancreatic  $\beta$  cells are considered to be useful for developing medicines, but their structures and functions have not been elucidated as yet.

#### [Disclosure of Invention]

Employing a cell in which a cDNA coding for orphan G protein-coupled receptor protein, phGR3 has been expressed by a suitable means and using measurement of a specific cell stimulation activity exemplified by a signal transduction activity as an indicator, the inventors of the present invention succeeded in

screening a polypeptide derived from bovine, human, rat and determined their amino acid sequences and nucleotide sequences.

Furthermore, the inventors found that the ligand polypeptide has prolactin secretion and/or placental function(s).

The present invention, therefore, relates to

- (1) an agent for modulating prolactin secretion which comprises a ligand polypeptide, or a salt thereof, for a G protein-coupled receptor protein,
- (2) an agent as described in (1) above, wherein the ligand polypeptide is a polypeptide comprising an amino acid sequence represented by SEQ ID NO: 73 or a substantial equivalent thereto, or its amide or ester, or a salt thereof,
- (3) an agent as described in (2) above, wherein the polypeptide comprising an amino acid sequence represented by SEQ ID NO: 73 is a polypeptide comprising an amino acid sequence represented by SEQ ID NO: 5, 8, 47, 50, 61 or 64,
- (4) an agent as described in (1) above, which is for promoting prolactin secretion,
- (5) an agent as described in (1) above, which is for inhibiting prolactin secretion,
- (6) an agent as described in (4) above, which is for treating or preventing hypoovarianism, gonocyst cacogenesis, menopausal syndrome, or euthyroid hypometabolism,
- (7) An agent as described in (5) above, which is for treating or preventing pituitary adenomatosis, brain tumor, emmeniopathy, autoimmune disease, prolactinoma, infertility, impotence, amenorrhea, galactorrhea, acromegaly, Chiari-Frommel syndrome, Argonz-del Castillo syndrome, Forbes-Albright syndrome, lymphoma, Sheehan syndrome or dyszoospermia,
- (8) An agent for modulating placental function, which

comprises a ligand polypeptide, or a salt thereof, for a G protein-coupled receptor protein,

(9) An agent as described in (8) above, which is for treating or preventing choriocarcinoma, hydatid mole, irruption mole, abortion, unthrifty fetus, abnormal saccharometabolism, abnormal lipidmetabolism or oxytocia,

(10) An agent as described in (4) above, which is for promoting lactation of domestic mammal,

(11) An agent as described in (4) above, which is for an aphrodisiac,

(12) An agent for diagnosing function of prolactin secretion, which comprises a ligand polypeptide, or a salt thereof, for a G protein-coupled receptor protein,

(13) Use of a ligand polypeptide, or a salt thereof, for a G protein-coupled receptor protein for manufacture of a medicament for modulating prolactin secretion,

(14) A method for modulating prolactin secretion in a mammal, which comprises administering to said mammal a ligand polypeptide, or a salt thereof, for a G protein-coupled receptor protein,

(15) Use of a ligand polypeptide, or a salt thereof, for a G protein-coupled receptor protein for manufacture of a medicament for modulating placental function, and

(16) A method for modulating placental function in a mammal, which comprises administering to said mammal a ligand polypeptide, or a salt thereof, for a G protein-coupled receptor protein, and so on.

#### [Brief Description of the Drawings]

Fig. 1 shows the nucleotide sequence of the human pituitary-derived G protein-coupled receptor protein cDNA fragment harbored in cDNA clone p19P2 isolated by PCR using human pituitary-derived cDNA and the amino

acid encoded by the nucleotide sequence. The primer used for sequencing was -21M13. The underscored region corresponds to the synthetic primer.

Fig. 2 shows the nucleotide sequence of the human  
5 pituitary-derived G protein-coupled receptor protein  
cDNA fragment harbored in cDNA clone p19P2 isolated by  
PCR using human pituitary-derived cDNA and the amino  
acid sequence encoded thereby. The primer used for  
sequencing was M13RV-N (Takara). The underscored  
10 region corresponds to the synthetic primer.

Fig. 3 shows a partial hydrophobic plot of the  
protein encoded by the human pituitary-derived G  
protein-coupled receptor protein cDNA fragment harbored  
in p19P2 constructed according to the amino acid  
15 sequence shown in Fig. 1.

Fig. 4 shows a partial hydrophobic plot of the  
protein encoded by the human pituitary-derived G  
protein-coupled receptor protein cDNA fragment harbored  
in p19P2 constructed according to the amino acid  
20 sequence shown in Fig. 2.

Fig. 5 is a diagram comparing the partial amino  
acid sequence of the protein encoded by the human  
pituitary-derived G protein-coupled receptor protein  
cDNA fragment harbored in p19P2 as shown in Figs. 1 and  
25 2 with the known G protein-coupled receptor protein  
S12863. The shadowed region represents the region of  
agreement. The 1 to 9 amino acid sequence of p19P2  
corresponds to the 1 to 99 amino acid sequence of Fig.  
1 and the 156 to 230 amino acid sequence corresponds to  
30 the 1 to 68 amino acid sequence of Fig. 2.

Fig. 6 shows the nucleotide sequence of the MIN6-  
derived G protein-coupled receptor protein cDNA  
fragment based on the nucleotide sequences of the MIN6-  
derived G protein-coupled receptor protein cDNA  
35 fragments harbored in the cDNA clones pG3-2 and pG1-10  
isolated by PCR using MIN6-derived cDNA and the amino

acid sequence encoded by the nucleotide sequence. The underscored region corresponds to the synthetic primer.

Fig. 7 is a diagram comparing the partial amino acid sequence encoded by pG3-2/pG1-10 of the MIN6-derived G protein-coupled receptor protein shown in Fig. 6 with the partial amino acid sequence of the protein encoded by p19P2 shown in Figs. 1 and 2. The shadowed region corresponds to the region of agreement. The 1 to 99 amino acid sequence of the protein encoded by p19P2 corresponds to the 1 to 99 amino acid sequence of Fig. 1 and the 156 to 223 amino acid sequence corresponds to the 1 to 68 amino acid sequence of Fig. 2. The 1 to 223 amino acid sequence of the protein encoded by pG3-2/pG1-10 corresponds to the 1 to 223 amino acid sequence of Fig. 6.

Fig. 8 is a partial hydrophobic plot of the MIN6-derived G protein-coupled receptor protein constructed according to the partial amino acid sequence shown in Fig. 6. Fig. 9 shows the entire nucleotide sequence of the human pituitary-derived G protein-coupled receptor protein cDNA harbored in the cDNA clone phGR3 isolated from a human pituitary-derived cDNA library by the plaque hybridization method using the DNA fragment inserted in p19P2 as a probe and the amino acid sequence encoded by the nucleotide sequence.

Fig. 10 shows the result of Northern blotting of human pituitary mRNA hybridized with radioisotope-labeled human pituitary cDNA clone phGR3.

Fig. 11 shows a hydrophobic plot of the protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA harbored in the phGR3 as constructed according to the amino acid sequence shown in Fig. 9.

Fig. 12 shows the nucleotide sequence of the MIN6-derived G protein-coupled receptor protein cDNA fragment harbored in the cDNA clone p5S38 isolated by

PCR using MIN6-derived cDNA and the amino acid sequence encoded by the nucleotide sequence. The underscored region corresponds to the synthetic primer.

Fig. 13 shows a diagram comparing the partial amino acid sequence of MIN6-derived G protein-coupled receptor protein encoded by p5S38 shown in Fig. 12 with the partial amino acid sequence of G protein-coupled receptor protein encoded by the cDNA fragment harbored in p19P2 as shown in Figs. 1 and 2 and the partial amino acid sequence of G protein-coupled receptor protein encoded by the nucleotide sequence generated from the nucleotide sequences of cDNA fragments contained in pG3-2 and pG1-10 shown in Fig. 6. The shadowed region represents the sequence region of agreement. The 1 to 144 amino acid sequence of the protein encoded by p5S38 corresponds to the 1 to 144 amino acid sequence of Fig. 12, the 1 to 99 amino acid sequence of the protein encoded by p19P2 corresponds to the 1 to 99 amino acid sequence of Fig. 1 and the 156 to 223 amino acid sequence corresponds to 1 to 68 amino acid sequence of Fig. 2. The 1 to 223 amino acid sequence of the protein encoded by pG3-2/pG1-10 corresponds to the 1 to 223 amino acid sequence of Fig. 6.

Fig. 14 shows a partial hydrophobic plot of the protein encoded by the MIN6-derived G protein-coupled receptor protein cDNA harbored in p5S38 as constructed according to the partial amino acid sequence shown in Fig. 12.

Fig. 15 shows the results of the following analysis. Thus, RT-PCR was carried out to confirm the expression of mRNA in CHO cells transfected by pAKKO-19P2. Lanes 1-7 represent the results obtained by performing PCRs using serial dilutions of pAKKO-19P2 for comparison, i.e. the 10  $\mu$ l/ml stock solution (lane 1), 1/2 dilution (lane 2), 1/4 dilution (lane 3), 1/64 dilution (Lane 4),

1/256 dilution (lane 5), 1/1024 dilution (lane 6), and 1/4096 dilution (lane 7) of the plasmid as templates, and analyzing the reaction mixtures by 1.2% agarose gel electrophoresis. Lanes 8 through 11 are the results obtained by performing PCRs using a 1/10 dilution (lane 8), a 1/100 dilution (lane 9), and a 1/1000 dilution (lane 10) of the cDNA prepared from the CHO-19P2 cell line as templates and subjecting the respective reaction mixtures to electrophoresis. Lane 11 was obtained by performing PCR using a template obtained by carrying out cDNA synthesis without reverse transcriptase and subjecting the PCR reaction product to electrophoresis. Lanes 12 and 13 were obtained by performing PCR using cDNAs prepared from mock CHO cells with and without addition of reverse transcriptase, respectively, as templates and subjecting the respective reaction products to electrophoresis. M represents the DNA size marker. The lanes at both ends were obtained by electrophoresing 1  $\mu$ l of  $\lambda$ /Sty I digest (Nippon Gene) and the second lane from right was obtained with 1  $\mu$ l of  $\phi$  X 174/Hinc II digest (Nippon Gene). The arrowmark indicates the position of the band amplified by PCR of about 400 bp.

Fig. 16 shows the activity of the crude ligand peptide fraction extracted from rat whole brain to promote release of arachidonic acid metabolites from CHO-19P2 cells. The arachidonic acid metabolite releasing activity was expressed as % of the amount of [ $^3$ H] arachidonic acid metabolites released in the presence of the crude ligand polypeptide fraction with the amount of [ $^3$ H] arachidonic acid metabolites released in the presence of 0.05% BAS-HABB being taken as 100%. The activity to promote release of arachidonic acid metabolites from the CHO-19P2 cell line was detected in a 30% CH<sub>3</sub>CN fraction.

Fig. 17 shows the activity of the crude ligand

polypeptide fraction extracted from bovine hypothalamus to promote release of arachidonic acid metabolites from CHO-19P2 cells. The arachidonic acid metabolite release-promoting activity was expressed as % of the amount of [ $^3\text{H}$ ] arachidonic acid metabolites released in the presence of the crude ligand polypeptide fraction with the amount of [ $^3\text{H}$ ] arachidonic acid metabolites released in the presence of 0.05% BAS-HABB being taken as 100%. The activity to promote release of arachidonic acid metabolites from the CHO-19P2 cell line was detected in a 30%  $\text{CH}_3\text{CN}$  fraction just as in the crude ligand polypeptide fraction from rat whole brain.

Fig. 18 shows the activity of the fraction purified with the reversed-phase column C18 218TP5415 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The active fraction from RESOURCE S was fractionated on C18 218TP5415. Thus, chromatography was carried out at a flow rate of 1 ml/min. on a concentration gradient of 20%-30%  $\text{CH}_3\text{CN}$  /0.1% TFA/ $\text{H}_2\text{O}$ , the eluate was collected in 1 ml fractions, and each fraction was lyophilized. Then, the activity of each fraction to specifically promote release of arachidonic acid metabolites from the CHO-19P2 cell line was determined. As a result, the activity was fractionated into 3 fractions (designated, in the order of elution, as P-1, P-2, and P-3).

Fig. 19 shows the activity of the fraction purified with the reversed-phase column diphenyl 219TP5415 to specifically promote arachidonic acid metabolite release from CHO-19P2 cells. The P-3 active fraction from C18 218TP5415 was fractionated on diphenyl 219TP5415. The chromatography was carried out at a flow rate of 1 ml/min. on a concentration gradient of 22%-25%  $\text{CH}_3\text{CN}$  /0.1% TFA/  $\text{H}_2\text{O}$ , the eluate was collected in 1 ml fractions, and each fraction was lyophilized.



Then, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells in each fraction was determined. As a result, the activity converged in a single peak.

5        Fig. 20 shows the activity of the fraction purified by reversed-phase column  $\mu$  RPC C2/C18 SC 2.1/10 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The peak active fraction from diphenyl 219TP5415 was fractionated on  $\mu$  10        RPC C2/C18 SC 2.1/10. The chromatography was carried out at a flow rate of 100  $\mu$  l/min. on a concentration gradient of 22%-23.5%  $\text{CH}_3\text{CN}$  /0.1% TFA/  $\text{H}_2\text{O}$ , the eluate was collected in 100  $\mu$  l fractions, and each fraction was lyophilized. Then, the activity to specifically 15        promote release of arachidonic acid metabolites from CHO-19P2 cells in each fraction was determined. As a result, the activity was found as two peaks of apparently a single substance (peptide).

      Fig. 21 shows the activity of the P-2 fraction 20        purified by reversed-phase column  $\mu$ RPC C2/C18 SC 2.1/10 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The chromatography was carried out at a flow rate of 100  $\mu$  l/min. on a concentration gradient of 21.5%-23.0%  $\text{CH}_3\text{CN}$  /0.1% 25        TFA/distilled  $\text{H}_2\text{O}$ , the eluate was collected in 100  $\mu$  l fractions, and each fraction was lyophilized. Then, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells in each fraction was determined. As a result, the 30        activity was found as a peak of apparently a single substance.

      Fig. 22 shows the nucleotide sequence of bovine hypothalamus ligand polypeptide cDNA fragment as derived from the nucleotide sequence of the bovine 35        hypothalamus-derived ligand polypeptide cDNA fragment which specifically promotes release of arachidonic acid

metabolites from CHO-19P2 cells as harbored in a cDNA clone isolated by PCR using bovine hypothalamus-derived cDNA and the amino acid sequence encoded by said nucleotide sequence. The region indicated by the arrowmark corresponds to the synthetic primer.

Fig. 23 shows the nucleotide sequence of the bovine hypothalamus-derived ligand polypeptide cDNA fragment generated according to the nucleotide sequence of the bovine hypothalamus-derived ligand polypeptide cDNA fragment which specifically promotes release of arachidonic acid metabolites from CHO-19P2 cells as harbored in a cDNA clone isolated by PCR using bovine hypothalamus-derived cDNA and the amino acid sequence encoded by said nucleotide sequence. The region indicated by the arrowmark corresponds to the synthetic primer.

Fig. 24 shows the amino acid sequences (a) and (b) of the bovine hypothalamus-derived ligand polypeptides which specifically promote release of arachidonic acid metabolites from CHO-19P2 cells and the cDNA sequence coding for the full coding region of the ligand polypeptides defined by SEQ ID NO:1 and SEQ ID NO:44.

Fig. 25 shows the concentration-dependent activity of synthetic ligand polypeptide (19P2-L31) to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The synthetic peptide was dissolved in degassed distilled H<sub>2</sub>O at a final concentration of 10<sup>-3</sup>M and diluted with 0.05% BSA-HBSS to concentrations of 10<sup>-12</sup>M-10<sup>-6</sup>M. The arachidonic acid metabolite releasing activity was expressed in the measured radioactivity of [<sup>3</sup>H] arachidonic acid metabolites released in the supernatant when the dilution was added to the cells. As a result, the activity of 19P2-31 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was found in a concentration-dependent manner.

Fig. 26 shows the concentration-dependent activity of synthetic ligand polypeptide (19P2-L31(O)) to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The synthetic ligand peptide was dissolved in degassed distilled H<sub>2</sub>O at a final concentration of 10<sup>-3</sup>M and diluted with 0.05% BSA-HBSS to concentrations of 10<sup>-12</sup>M-10<sup>-6</sup>M. The arachidonic acid metabolite releasing activity was expressed in the measured radioactivity of [<sup>3</sup>H] arachidonic acid metabolites released in the supernatant when the dilution was added to the cells. As a result, the activity of 19P2-L31(O) to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was found in a dose-dependent manner.

Fig. 27 shows the activity of synthetic ligand polypeptide 19P2-L20 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The synthetic peptide was dissolved in degassed distilled H<sub>2</sub>O at a final concentration of 10<sup>-3</sup>M and diluted with 0.05% BSA-HBSS to concentrations of 10<sup>-12</sup>M-10<sup>-6</sup>M. The arachidonic acid metabolite releasing activity was expressed in the measured radioactivity of [<sup>3</sup>H] arachidonic acid metabolites released in the supernatant when the dilution was added to the cells. As a result, the activity of 19P2-L20 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was found in a dose-dependent manner.

Fig. 28 shows the 1.2% agarose gel electrophoregram of the DNA fragments of the phages cloned from a bovine genomic library as digested with restriction enzymes BamHI(B) and SalI(S). As the DNA size marker (M), StyI digests of  $\lambda$  phage DNA were used. In lane B, two bands derived from the vector were detected in positions between the first (19,329 bp) and second (7.743 bp) marker bands, as well as 3 bands derived from the inserted fragment between the third (6,223 bp) and 5th

(3,472 bp) bands. In lane S, two bands derived from the vector were similarly detected but due to the overlap of the band of the inserted fragment, the upper band is thicker than the band in lane B.

5        Fig. 29 shows the nucleotide sequence around the coding region as decoded from bovine genomic DNA. The 1st to 3rd bases (ATG) correspond to the translation start codon and the 767th to 769th bases (TAA) correspond to the translation end codon.

10        Fig. 30 shows a comparison between the nucleotide sequence (genome) around the coding region as deduced from bovine genomic DNA and the nucleotide sequence (cDNA) of bovine cDNA cloned by PCR. The sequence region of agreement is indicated by shading. As to the  
15        101st to 572nd region, there is no corresponding region in the nucleotide sequence of cDNA, indicating that it is an intron.

      Fig. 31 shows the translation of the amino acid sequence encoded after elimination of the intron from  
20        the nucleotide sequence around the coding region as decoded from bovine genomic DNA.

      Fig. 32 shows the full-length amino acid sequence and the cDNA sequence coding for the full coding region of rat ligand polypeptide.

25        Fig. 33 shows amino acid sequence of bovine ligand polypeptide and the nucleotide sequences of DNAs coding for bovine polypeptide and rat polypeptide. The arrowmark indicates the region corresponding to the synthetic primer.

30        Fig. 34 shows the full-length amino acid sequence and the sequence of cDNA coding for the full coding region of human ligand polypeptide.

      Fig. 35 shows a comparison of the amino acid sequences in the translation region of bovine ligand  
35        polypeptide, rat ligand polypeptide, and human ligand polypeptide.

Fig. 36 shows the results of a receptor binding experiment with an iodine-labeled ligand polypeptide in living cells.

Fig. 37 shows the arachidonic acid metabolite releasing activity of the ligand polypeptide in CHO-19P2-9 and CHO-UHR1.

Fig. 38 shows the results of RT-PCR assays of UHR-1 expressed in rat tissues. Each value is the mean  $\pm$  S.E.M. of 3 experiments.

Fig. 39 shows the results of RT-PCR assays of the ligand polypeptide expressed in rat tissues. Each value is the mean  $\pm$  S.E.M. of 3 experiments.

Fig. 40 shows the influence of the ligand polypeptide on the glucose-induced plasma insulin concentration determined by radioimmunoassay.

Fig. 41 shows the measured motor activity of mice treated with 10 nmol of the ligand polypeptide. (a): spontaneous motor activity, (b): rearing

Fig. 42 shows the measured motor activity of mice treated with 1 nmol of the ligand polypeptide. (a): spontaneous motor activity, (b): rearing

Fig. 43 shows the measured motor activity of mice treated with 0.1 nmol of the ligand polypeptide. (a): spontaneous motor activity, (b): rearing

Fig. 44 shows the measured motor activity of mice treated with 0.01 nmol of the ligand polypeptide. (a): spontaneous motor activity, (b): rearing

Fig. 45 shows the change in the body temperature of mice upon administration of the ligand polypeptide into the cerebral ventricle 15 hours following subcutaneous administration of 3 mg/kg reserpine. The single asterisk \* stands for  $p < 0.05$  and the double asterisk \*\* for  $p < 0.01$ .

Fig. 46 shows a schematic diagram showing a microinjection cannula inserted into the area postrema (AP) at an angle of 20 degrees.

Fig. 47 shows a typical example of pulse wave and mean blood pressure following injection of the ligand polypeptide into AP [Conscious rat, 10 nmol at a flow rate of 1  $\mu$ l/min].

5        Fig. 48 shows the plasma GH level following administration of the ligand polypeptide 50 nmol into the third ventricle of rats under pentobarbital anesthesia.

10       Fig. 49 shows the plasma GH level following administration of the ligand polypeptide into the third ventricle.

To unrestrained conscious rats, the ligand polypeptide or PBS was administered into the third ventricle following intraatrial injection of GHRH 5  $\mu$ g/kg. The point of time at which the polypeptide was administered was reckoned as 0 min. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ .

Fig. 50 shows the relationship of ligand polypeptide antiserum with absorbance.

20       Fig. 51 shows the results of determination of arachidonic acid metabolite releasing activity of the anti-ligand polypeptide polyclonal antibody.

Fig. 52 shows the nucleotide sequence of the full coding region of rat UHR-1 constructed on the expression vector pAKKO-UHR-1 and the amino acid sequence encoded thereby.

Fig. 53 shows the nucleotide sequence of the inserted fragment of plasmid pmGB3. → indicates the sequence corresponding to the primer.

30       Fig. 54 shows the predicted cDNA and translated protein based on the nucleotide sequence of plasmid pmGB3. → indicates the sequence corresponding to the primer. The sequence flanked by ↓ ↓ is the sequence predicted to be an intron.

35       Fig. 55 shows the change in prolactin release from rat pituitary RC-4B/C cells upon addition of

ligand polypeptide 19P2-L31.

Fig. 56 shows the change in prolactin secretion from primary cultured rat pituitary cells upon addition of ligand polypeptide 19P2-L31.

5 Fig. 57 shows the time course of expression of UHR-1 gene in the rat placenta described in Example 48.

Fig. 58 shows the time course of plasma prolactin concentration after administration of 19P2-L31 in unrestrained male rats.  $\ast=p<0.05$ . Each value is the mean  $\pm$  S.E.M. of 3-4 experiments.

10 Fig. 59 shows the time course of plasma prolactin concentration after administration of 19P2-L31 in unrestrained female rats.  $\ast=p<0.05$ . Each value is the mean  $\pm$  S.E.M. of 3-4 experiments.

15 Fig. 60 shows the time course of plasma prolactin concentration was determined among the sexual cycle.

[Best Mode for Carrying Out the Invention]

In the specification and drawings of the present application, the abbreviations used for bases (nucleotides), amino acids and so forth are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature or those conventionally used in the art. Examples thereof are given below. Amino acids for which optical isomerism is possible are, unless otherwise specified, in the L form.

DNA : Deoxyribonucleic acid

cDNA : Complementary deoxyribonucleic acid

A : Adenine

30 T : Thymine

G : Guanine

C : Cytosine

RNA : Ribonucleic acid

mRNA : Messenger ribonucleic acid

35 dATP : Deoxyadenosine triphosphate

dTTP : Deoxythymidine triphosphate

	dGTP : Deoxyguanosine triphosphate
	dCTP : Deoxycytidine triphosphate
	ATP : Adenosine triphosphate
	EDTA : Ethylenediamine tetraacetic acid
5	SDS : Sodium dodecyl sulfate
	EIA : Enzyme Immunoassay
	G, Gly: Glycine (or Glycyl)
	A, Ala: Alanine (or Alanyl)
	V, Val: Valine (or Valyl)
10	L, Leu: Leucine (or Leucyl)
	I, Ile: Isoleucine (or Isoleucyl)
	S, Ser: Serine (or Seryl)
	T, Thr: Threonine (or Threonyl)
	C, Cys: Cysteine (or Cysteinyl)
15	M, Met: Methionine (or Methionyl)
	E, Glu: Glutamic acid (or Glutamyl)
	D, Asp: Aspartic acid (or Aspartyl)
	K, Lys: Lysine (or Lysyl)
	R, Arg: Arginine (or Arginyl)
20	H, His: Histidine (or Histidyl)
	F, Phe: Phenylalanine (or Phenylalanyl)
	Y, Tyr: Tyrosine (or Tyrosyl)
	W, Trp: Tryptophan (or Tryptophanyl)
	P, Pro: Proline (or Prolyl)
25	N, Asn: Asparagine (or Asparaginyl)
	Q, Gln: Glutamine (or Glutaminyl)
	pGlu: Pyroglutamic acid (or Pyroglutamyl)
	Me: Methyl
	Et: Ethyl
30	Bu: Butyl
	Ph: Phenyl
	TC: Thiazolidinyl-4(R)-carboxamide

In this specification, substitutions, protective groups and reagents commonly used are indicated by the following abbreviations:

BHA : benzhydramine



- pMBHA : p-methylbenzhydramine  
Tos : p-toluenesulfonyl  
CHO : formyl  
HONB : N-hydroxy-5-norbornene-2,3-dicarboxyimide  
5 OcHex : cyclohexyl ester  
Bzl : benzyl  
Cl<sub>2</sub>-Bzl : dichloro-benzyl  
Bom : benzyloxymethyl  
Br-Z : 2-bromobenzyloxycarbonyl  
10 Boc : t-butoxycarbonyl  
DCM : dichloromethane  
HOBt : 1-hydroxybenztriazole  
DCC : N,N'-dicyclohexylcarbodiimide  
TFA : trifluoro acetate  
15 DIEA : diisopropylethylamine  
Fmoc : N-9-fluorenylmethoxycarbonyl  
DNP : dinitrophenyl  
Bom : t-butoxymethyl  
Trt : trityl  
20 As used herein the term "substantial equivalent(s)" means that the activity of the protein, e.g., nature of the binding activity of the ligand and the receptor and physical characteristics are substantially the same. Substitutions, deletions or insertions of amino acids  
25 often do not produce radical changes in the physical and chemical characteristics of a polypeptide, in which case polypeptides containing the substitution, deletion, or insertion would be considered to be substantially equivalent to polypeptides lacking the substitution,  
30 deletion, or insertion.  
Substantially equivalent substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs.  
(1) The non-polar (hydrophobic) amino acids include  
35 alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. (2) The

polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. (3) The positively charged (basic) amino acids include arginine, lysine and histidine. (4) The  
5 negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

The amino acids being comprised the ligand polypeptide of the present invention may form D-form or L-form, but usually form L-form.

10 The ligand polypeptide according to the present invention is a polypeptide which is capable of binding to G protein-coupled receptor protein and comprising an amino acid sequence represented by SEQ ID NO:73 or its substantial equivalent thereto, or its amide or ester,  
15 or a salt thereof (hereinafter sometimes referred to briefly as the ligand polypeptide or the polypeptide).

In SEQ ID NO:73, Xaa at 10th position is Ala or Thr; Xaa at 11th position is Gly or Ser; and Xaa at 21th position is H, Gly, or GlyArg.

20 Preferable example of the amino acid sequence represented by SEQ ID NO:73 is the amino acid sequence represented by SEQ ID NO:5, 8, 47, 50, 61 or 64. Among them, the amino acid sequence represented by SEQ ID NO:61 or 64 is more preferable. Further, the amino acid  
25 sequence represented by SEQ ID 64 is more preferable.

The above ligand polypeptide of the present invention includes any polypeptides derived from any tissues, e.g. pituitary gland, pancreas, brain, kidney, liver, gonad, thyroid gland, gall bladder, bone marrow,  
30 adrenal gland, skin, muscle, lung, digestive canal, blood vessel, heart, etc.; or cells of man and other warm-blooded animals, e.g. guinea pig, rat, mouse, swine, sheep, bovine, monkey, etc. and comprising an amino acid sequence represented by SEQ ID NO:73,  
35 preferably the amino acid sequence represented by SEQ ID NO:5, 8, 47, 50, 61 or 64, or its substantial

equivalent thereto. For example, in addition to the protein comprising the amino acid sequence of SEQ ID NO:73, preferably the amino acid sequence represented by SEQ ID NO:5, 8, 47, 50, 61 or 64, the ligand polypeptide of the present invention includes the protein comprising an amino acid sequence having a homology of about 50-99.9%, preferably 70-99.9%, more preferably 80-99.9% and especially preferably 90-99.9% to the amino acid sequence of SEQ ID NO:73, preferably the amino acid sequence represented by SEQ ID NO:5, 8, 47, 50, 61 or 64, and having qualitatively substantially equivalent activity to the protein comprising the amino acid sequence of SEQ ID NO:73, preferably the amino acid sequence represented by SEQ ID NO:5, 8, 47, 50, 61 or 64. The term "substantially equivalent" means the nature of the receptor-binding activity, signal transduction activity and the like is equivalent. Thus, it is allowable that even differences among grades such as the strength of receptor binding activity and the molecular weight of the polypeptide are present.

To be more specific, the ligand polypeptide of the present invention includes the polypeptide derived from the rat whole brain, bovine hypothalamus, or human whole brain and comprising the amino acid sequence of SEQ ID NO:73. In addition, the ligand polypeptide of the present invention includes the polypeptides which comprises substantially equivalent polypeptides such as (1) polypeptides wherein 1 to 15, preferably 1 to 10, and more preferably 1 to 5 amino acid residues are deleted from the amino acid sequence of SEQ ID NO:73, (2) polypeptides wherein 1 to 80, preferably 1 to 50, more preferably 1 to 10 amino acid residues are added to the amino acid sequence of SEQ ID NO:73, (3) polypeptides wherein 1 to 15, preferably 1 to 10, more preferably 1 to 5 amino acid residues are

substituted with one or more other amino acid residues of the amino acid sequence of SEQ ID NO:73, or

(4) polypeptide wherein the amino acid, especially its side chain, of the polypeptide of the above (1) to (3) is modified, or its amide thereof, or its ester thereof, or a salt thereof.

Among them, preferred is the polypeptide comprising the amino acid sequence of SEQ ID NO:73 and the polypeptide comprising the amino acid sequence which a peptide of SEQ ID NO:74 is added to the N-terminus of the polypeptide comprising the amino acid sequence of SEQ ID NO:73.

The ligand polypeptide of the present invention can be changed or mutated by substitution, deletion, addition or modification as mentioned above (1) to (4), to a polypeptide which is stable against heat or proteases, or a polypeptide whose physiological function is activated.

The ligand polypeptide or an amide thereof, or an ester thereof, or a salt thereof includes the changed or mutated polypeptide mentioned above.

The peptides described in this specification, the left ends are the N-terminus (amino terminus) and the right end is the C-terminus (carboxyl terminus) according to the convention of the peptide indication.

Furthermore, the polypeptide or partial peptide of the present invention includes those wherein the N-terminal side of Gln is cleaved in vivo to form pyroglutamyl peptide.

While the C-terminus of the polypeptide of the present invention, for example the polypeptide comprising the amino acid sequence of SEQ ID NO:73, is usually carboxyl (-COOH) or carboxylate (-COO-), it may be amide (-CONH<sub>2</sub>) or ester (-COOR) form. The ester residue R includes a C<sub>1-6</sub> alkyl group such as methyl, ethyl, n-propyl, isopropyl, n-butyl, etc., a C<sub>3-8</sub>

cycloalkyl group such as cyclopentyl, cyclohexyl, etc.,  
a C<sub>6-12</sub> aryl group such as phenyl,  $\alpha$ -naphthyl, etc.,  
and a C<sub>7-14</sub> aralkyl group such as a phenyl-C<sub>1-2</sub> alkyl  
group, e.g. benzyl, phenethyl, benzhydryl, etc. or an  
5  $\alpha$ -naphthyl-C<sub>1-2</sub> alkyl, e.g.  $\alpha$ -naphthylmethyl etc. In  
addition, the ester may be a pivaloyloxymethyl ester  
which is broadly used for oral administration.

When the polypeptide of the present invention, for  
example the polypeptide comprises the amino acid  
10 sequence of SEQ ID NO:73, has a carboxyl or carboxylate  
group in any position other than the C-terminus, the  
corresponding amide or ester are also included in the  
concept of the polypeptide of the present invention.  
The ester mentioned just above includes the esters  
15 mentioned for the C-terminus.

The preferred ligand polypeptide of the present  
invention is a peptide which the C-terminus is amidated.  
Especially preferred is a polypeptide comprising the  
amino acid sequence of SEQ ID NO:5, 8, 47, 50, 61 or 64  
20 which the C-terminus is amidated.

The salt of polypeptide of the present invention  
includes salts with physiologically acceptable bases,  
e.g. alkali metals or acids such as organic or  
inorganic acids, and is preferably a physiologically  
25 acceptable acid addition salt. Examples of such salts  
are salts thereof with inorganic acids, e.g.  
hydrochloric acid, phosphoric acid, hydrobromic acid or  
sulfuric acid, etc. and salts thereof with organic  
acids, e.g. acetic acid, formic acid, propionic acid,  
30 fumaric acid, maleic acid, succinic acid, tartaric acid,  
citric acid, malic acid, oxalic acid, benzoic acid,  
methanesulfonic acid or benzenesulfonic acid, etc.

The ligand polypeptide or amide or ester, or a salt  
thereof of the present invention may be  
35 (1) manufactured from the tissues or cells of warm-  
blooded animals inclusive of human by purifying

techniques or

(2) manufactured by the peptide synthesis as described hereinafter.

(3) Moreover, it can be manufactured by culturing a transformant carrying a DNA coding for the polypeptide as described hereinafter.

(1) In the production from the tissues or cells of human or other warm-blooded animals, the ligand polypeptide can be purified and isolated by a process which comprises homogenizing the tissue or cells of human or other warm-blooded animal, extracting the homogenate with an acid, for instance, and subjecting the extract to a combination of chromatographic procedures such as reversed-phase chromatography, ion-exchange chromatography, affinity chromatography, etc.

(2) As mentioned above, the ligand polypeptide in the present invention can be produced by the per se known procedures for peptide synthesis. The methods for peptide synthesis may be any of a solid-phase synthesis and a liquid-phase synthesis. Thus, the objective peptide can be produced by condensing a partial peptide or amino acid capable of constituting the protein with the residual part thereof and, when the product has a protective group, the protective group is detached whereupon a desired peptide can be manufactured. The known methods for condensation and deprotection includes the procedures described in the following literature (1)-(5).

(1) M. Bodanszky and M. A. Ondetti, Peptide Synthesis, Interscience Publishers, New York, 1966.

(2) Schroeder and Luebke, The Peptide, Academic Press, New York, 1965

(3) Nobuo Izumiya et al., Fundamentals and Experiments in Peptide Synthesis, Maruzen, 1975

(4) Haruaki Yajima and Shumpei Sakakibara, Biochemical Experiment Series 1, Protein Chemistry IV,

205, 1977

(5) Haruaki Yajima (ed.), Development of Drugs-Continued, 14, Peptide Synthesis, Hirokawa Shoten

After the reaction, the protein can be purified and isolated by a combination of conventional purification techniques such as solvent extraction, column chromatography, liquid chromatography, and recrystallization. Where the protein isolated as above is a free compound, it can be converted to a suitable salt by the known method. Conversely where the isolated product is a salt, it can be converted to the free peptide by the known method.

The amide of polypeptide can be obtained by using a resin for peptide synthesis which is suited for amidation. The resin includes chloromethyl resin, hydroxymethyl resin, benzhydrylamine resin, aminomethyl resin, 4-benzyloxybenzyl alcohol resin, 4-methylbenzhydrylamine resin, PAM resin, 4-hydroxymethylmethylphenylacetamidomethyl resin, polyacrylamide resin, 4-(2',4'-dimethoxyphenylhydroxymethyl)phenoxy resin, 4-(2',4'-dimethoxyphenyl-Fmoc aminoethyl)phenoxy resin, and so on. Using such a resin, amino acids whose  $\alpha$ -amino groups and functional groups of side-chain have been suitably protected are condensed on the resin according to the sequence of the objective peptide by various condensation techniques which are known per se. At the end of the series of reactions, the peptide or the protected peptide is removed from the resin and the protective groups are removed to obtain the objective polypeptide.

For the condensation of the above-mentioned protected amino acids, a variety of activating reagents for peptide synthesis can be used but a carbodiimide compound is particularly suitable. The carbodiimide includes DCC, N,N'-diisopropylcarbodiimide, and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide. For

activation with such a reagent, a racemization inhibitor additive, e.g. HOBt and the protected amino acid are directly added to the resin or the protected amino acid pre-activated as symmetric acid anhydride, HOBt ester, or HOObt ester is added to the resin. The solvent for the activation of protected amino acids or condensation with the resin can be properly selected from among those solvents which are known to be useful for peptide condensation reactions. For example, N,N-dimethylformamide, N-methylpyrrolidone, chloroform, trifluoroethanol, dimethyl sulfoxide, DMF, pyridine, dioxane, methylene chloride, tetrahydrofuran, acetonitrile, ethyl acetate, or suitable mixtures of them can be mentioned. The reaction temperature can be selected from the range hitherto-known to be useful for peptide bond formation and is usually selected from the range of about  $-20^{\circ}\text{C}$  -  $50^{\circ}\text{C}$ . The activated amino acid derivative is generally used in a proportion of 1.5-4 fold excess. If the condensation is found to be insufficient by a test utilizing the ninhydrin reaction, the condensation reaction can be repeated to achieve a sufficient condensation without removing the protective group. If repeated condensation still fails to provide a sufficient degree of condensation, the unreacted amino group can be acetylated with acetic anhydride or acetylimidazole.

The protecting group of amino group for the starting material amino acid includes Z, Boc, tertiary-amyloxycarbonyl, isobornyloxycarbonyl, 4-methoxybenzyloxycarbonyl, Cl-Z, Br-Z, adamantyloxycarbonyl, trifluoroacetyl, phthalyl, formyl, 2-nitrophenylsulfenyl, diphenylphosphinothioyl, or Fmoc. The carboxy-protecting group that can be used includes but is not limited to the above-mentioned  $\text{C}_{1-6}$  alkyl,  $\text{C}_3$ , cycloalkyl and  $\text{C}_{7-14}$  aralkyl as well as 2-adamantyl, 4-nitrobenzyl, 4-methoxybenzyl, 4-chlorobenzyl, phenacyl,



benzyloxycarbonylhydrazido, tertiary-butoxycarbonylhydrazido, and tritylhydrazido.

The hydroxy group of serine and threonine can be protected by esterification or etherification. The group suited for said esterification includes carbon-derived groups such as lower alkanoyl groups, e.g. acetyl etc., aroyl groups, e.g. benzoyl etc., benzyloxycarbonyl, and ethoxycarbonyl. The group suited for said etherification includes benzyl, tetrahydropyranyl, and tertiary-butyl.

The protective group for the phenolic hydroxyl group of tyrosine includes Bzl, C<sub>12</sub>-Bzl, 2-nitrobenzyl, Br-Z, and tertiary-butyl.

The protecting group of imidazole for histidine includes Tos, 4-methoxy-2,3,6-trimethylbenzenesulfonyl, DNP, benzyloxymethyl, Bum, Boc, Trt, and Fmoc.

The activated carboxyl group of the starting amino acid includes the corresponding acid anhydride, azide, and active esters, e.g. esters with alcohols such as pentachlorophenol, 2,4,5-trichlorophenol, 2,4-dinitrophenol, cyanomethyl alcohol, p-nitrophenol, HONB, N-hydroxysuccinimide, N-hydroxyphthalimide, HOBt, etc. The activated amino group of the starting amino acid includes the corresponding phosphoramidate.

The method for elimination of protective groups includes catalytic reduction using hydrogen gas in the presence of a catalyst such as palladium black or palladium-on-carbon, acid treatment with anhydrous hydrogen fluoride, methanesulfonic acid, trifluoromethanesulfonic acid, trifluoroacetic acid, or a mixture of such acids, base treatment with diisopropylethylamine, triethylamine, piperidine, piperazine, reduction with sodium metal in liquid ammonia. The elimination reaction by the above-mentioned acid treatment is generally carried out at a temperature of -20 °C - 40 °C and can be conducted

advantageously with addition of a cation acceptor such as anisole, phenol, thioanisole, m-cresol, p-cresol, dimethyl sulfide, 1,4-butanedithiol, 1,2-ethanedithiol. The 2,4-dinitrophenyl group used for protecting the imidazole group of histidine can be eliminated by treatment with thiophenol, while the formyl group used for protecting the indole group of tryptophan can be eliminated by alkali treatment with dilute sodium hydroxide solution or dilute aqueous ammonia as well as the above-mentioned acid treatment in the presence of 1,2-ethanedithiol, 1,4-butanedithiol.

The method for protecting functional groups which should not take part in the reaction of the starting material, the protective groups that can be used, the method of removing the protective groups, and the method of activating the functional groups that are to take part in the reaction can all be selected from among the known groups and methods.

An another method for obtaining the amide form of the polypeptide comprises amidating the  $\alpha$ -carboxyl group of the C-terminal amino acid at first, then extending the peptide chain to the N-side until the desired chain length, and then selectively deprotecting the  $\alpha$ -amino group of the C-terminal peptide and the  $\alpha$ -carboxy group of the amino acid or peptide that is to form the remainder of the objective polypeptide and condensing the two fragments whose  $\alpha$ -amino group and side-chain functional groups have been protected with suitable protective groups mentioned above in a mixed solvent such as that mentioned hereinbefore. The parameters of this condensation reaction can be the same as described hereinbefore. From the protected peptide obtained by condensation, all the protective groups are removed by the above-described method to thereby provide the desired crude peptide. This crude peptide can be purified by known purification

procedures and the main fraction be lyophilized to provide the objective amidated polypeptide.

To obtain an ester of the polypeptide, the  $\alpha$ -carboxyl group of the C-terminal amino acid is condensed with a desired alcohol to give an amino acid ester and then, the procedure described above for production of the amide is followed.

The ligand polypeptide of the present invention, its amide or ester, or a salt thereof can be any peptide that has the same activities, e.g. pituitary function modulating activity, central nervous system function modulating activity, pancreatic function modulating activity, prolactin secretion modulating activity or placental function modulating activity, as the polypeptide which has an amino acid sequence of SEQ ID NO:73 or its substantial equivalent thereto. As such peptides, there can be mentioned peptides wherein 1 to 15 amino acids residues are deleted from the above-mentioned amino acid sequence of SEQ ID NO:73. To be specific, the peptide having an amino acid sequence corresponding to the 2nd to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 3rd to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 4th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 5th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 6th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 7th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 8th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 9th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide

corresponding to the 10th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 11th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide  
5 corresponding to the 12th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 13th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 14th to 21st positions of the  
10 amino acid sequence of SEQ ID NO:73, and the peptide corresponding to the 15th to 21st positions of the amino acid sequence of SEQ ID NO:73, can be mentioned as preferred examples. Moreover, the peptide having the amino acid sequence of SEQ ID NO:74 is also  
15 preferred.

Examples of the ligand polypeptide for the polypeptide comprises the amino acid sequence of SEQ ID NO:5, 8, 47, 50 or 61 each of which is an preferable example of the polypeptide comprising the amino acid  
20 sequence of SEQ ID NO:73, are the same as the cases of the polypeptide comprising the amino acid sequence of SEQ ID NO:73, mentioned above.

The DNA coding for the ligand polypeptide or a partial peptide thereof of the present invention may be  
25 any DNA comprising the nucleotide sequence encoding a polypeptide having an amino acid sequence of SEQ ID NO:73 or its substantial equivalent thereto. Furthermore, the DNA may be any of genomic DNA, genomic DNA library, tissue- or cell-derived cDNA, tissue- or  
30 cell-derived cDNA library, and synthetic DNA. The vector for such as library may be any of bacteriophage, plasmide, cosmide, and phagimide. Moreover, it can be directly amplified by the RT-PCR(reverse transcription PCR) method by using an RNA fraction may be prepared  
35 from a tissue or cells .

To be more specific, as the DNA coding for a

polypeptide derived from rat whole brain or bovine hypothalamus and comprising the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:44, the DNA comprising the nucleotide sequence of SEQ ID NO:2 can be exemplified.

5 In SEQ ID NO:2, R at 129th position represents G or A, and Y at 179th and 240th positions represents C or T. When Y at 179th position is C, the amino acid sequence of SEQ ID NO:1 is encoded, and when Y at 179th position is T, the amino acid sequence of SEQ ID NO:44 is encoded.

10 As the DNA coding for a bovine-derived polypeptide comprising the amino acid sequence of SEQ ID NO:3, 4, 5, 6, 7, 8, 9 or 10, a DNA comprising the nucleotide sequence of SEQ ID NO:11, 12, 13, 14, 15, 16, 17 or 18 can be exemplified. Here, R at 63th position of SEQ ID NO:11, 13, 14 or 15 and R at 29th position of SEQ ID NO:12, 16, 17, or 18 represent G or A.

20 As the DNA coding for a rat-derived polypeptide of SEQ ID NO:45, 47, 48, 49, 50, 51, or 52, a DNA comprising the nucleotide sequence of SEQ ID NO:46, 53, 54, 55, 56, 57, or 58 can be exemplified.

25 Furthermore, as the DNA coding for a human-derived peptide of SEQ ID NO:59, 61, 62, 63, 64, 65, or 66, a DNA comprising the nucleotide sequence of SEQ ID NO:60, 67, 68, 69, 70, 71, or 72 can be exemplified.

30 Among DNAs coding for the bovine-derived polypeptide comprising the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:44, the rat-derived polypeptide comprising the amino acid sequence of SEQ ID NO:45, or the human-derived polypeptide comprising the amino acid sequence of SEQ ID NO:59, DNA fragments comprising partial nucleotide sequences of 6 to 90, preferably 6 to 60, more preferably 9 to 30, and especially preferably 12 to 30 can be advantageously used as DNA probes as well.

35 The DNA coding for the ligand polypeptide or a

partial peptide thereof of the present invention can be produced by the following genetic engineering procedures.

(3) The DNA fully encoding the polypeptide of the present invention can be cloned either by PCR amplification using synthetic DNA primers having a partial nucleotide sequence of the polypeptide or partial peptide or by hybridization using the DNA inserted in a suitable vector and labeled with a DNA fragment comprising a part or full region of a human-derived polypeptide or a synthetic DNA. The hybridization can be carried out typically by the procedure described in Molecular Cloning (2nd ed., J. Sambrook et al., Cold Spring Harbor Lab. Press, 1989). When a commercial library is used, the instructions given in the accompanying manual can be followed.

The cloned DNA coding for the polypeptide or partial peptide can be used directly or after digestion with a restriction enzyme or addition of a linker depending on purposes. This DNA has ATG as the translation initiation codon at the 5' end and may have TAA, TGA, or TAG as the termination codon at the 3' end. The translation initiation and termination codons can be added by means of suitable DNA adapters.

An expression vector for the polypeptide or partial peptide can be produced by, for example (a) cutting out a target DNA fragment from the DNA for the polypeptide or partial peptide of the present invention and (b) ligating the target DNA fragment with the downstream side of a promoter in a suitable expression vector.

The vector may include plasmids derived from *Escherichia coli*, e.g., pBR322, pBR325, pUC12, pUC13, etc.; plasmids derived from *Bacillus subtilis*, e.g., pUB110, pTP5, pC194, etc.; plasmids derived from yeasts e.g., pSH19, pSH15, etc.; bacteriophages such as  $\lambda$  - phage, and animal virus such as retrovirus, vaccinia

virus and baculovirus.

According to the present invention, any promoter can be used as long as it is compatible with the host cell which is used for expressing a gene. When the  
5 host for the transformation is *E. coli*, the promoters are preferably *trp* promoters, *lac* promoters, *recA* promoters,  $\lambda$ PL promoters, *lpp* promoters, etc. When the host for the transformation is *Bacillus*, the promoters are preferably SPO1 promoters, SPO2 promoters, *penP*  
10 promoters, etc. When the host is a yeast, the promoters are preferably PHO5 promoters, PGK promoters, GAP promoters, ADH promoters, etc. When the host is an animal cell, the promoters include SV40-derived promoters, retrovirus promoters, metallothionein  
15 promoters, heat shock promoters, cytomegalovirus (CMV) promoters, SR  $\alpha$  promoters, etc. An enhancer can be effectively utilized for expression.

As required, furthermore, a host-compatible signal sequence is added to the N-terminal side of the  
20 polypeptide or partial peptide thereof. When the host is *E. coli*, the utilizable signal sequences may include alkaline phosphatase signal sequences, OmpA signal sequences, etc. When the host is *Bacillus*, they may include  $\alpha$ -amylase signal sequences, subtilisin signal  
25 sequences, etc. When the host is a yeast, they may include mating factor  $\alpha$  signal sequences, invertase signal sequences, etc. When the host is an animal cell, they may include insulin signal sequences,  $\alpha$ -interferon signal sequences, antibody molecule signal  
30 sequences, etc.

A transformant or transfectant is produced by using the vector thus constructed, which carries the polypeptide or partial peptide-encoding DNA of the present invention. The host may be, for example,  
35 *Escherichia* microorganisms, *Bacillus* microorganisms, yeasts, insect cells, animal cells, etc. Examples of

the Escherichia include Escherichia coli K12.DH1 [Proc. Natl. Acad. Sci. USA, Vol. 60, 160 (1968)], JM103 [Nucleic Acids Research, Vol. 9, 309 (1981)], JA221 [Journal of Molecular Biology, Vol. 120, 517 (1978)], HB101 [Journal of molecular Biology, Vol. 41, 459 (1969)], C600 [Genetics, Vol. 39, 440 (1954)], etc. Examples of the Bacillus microorganism are, for example Bacillus subtilis MI114 [Gene, Vol. 24, 255 (1983)], 207-21 [Journal of Biochemistry, Vol. 95, 76 (1984)], etc. The yeast may be, for example, Saccharomyces cerevisiae AH22, AH22R, NA87-11A, DKD-5D, 20B-12, etc. The insect may include a silkworm (Bombyx mori larva), [Maeda et al, Nature, Vol. 315, 592 (1985)] etc. The host animal cell may be, for example, monkey-derived cell line, COS-7, Vero, Chinese hamster ovary cell line (CHO cell), DHFR gene-deficient Chinese hamster cell line (dhfr CHO cell), mouse L cell, mouse myeloma cell, human FL, etc.

Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. Transformation of Escherichia microorganisms can be carried out in accordance with methods as disclosed in, for example, Proc. Natl. Acad. Sci. USA, Vol. 69, 2110 (1972), Gene, Vol. 17, 107 (1982), etc. Transformation of Bacillus microorganisms can be carried out in accordance with methods as disclosed in, for example, Molecular & General Genetics, Vol. 168, 111 (1979), etc. Transformation of the yeast can be carried out in accordance with methods as disclosed in, for example, Proc. Natl. Acad. Sci. USA, Vol. 75, 1929 (1978), etc. The insect cells can be transformed in accordance with methods as disclosed in, for example, Bio/Technology, 6, 47-55, 1988. The animal cells can be transformed by methods as disclosed in, for example, Virology, Vol. 52, 456, 1973, etc. The transformants or transfectants harboring the expression vector



carrying a polypeptide or partial peptide thereof encoding DNA are produced according to the aforementioned techniques.

5       Cultivation of the transformant (transfectant) in which the host is *Escherichia* or *Bacillus* microorganism can be carried out suitably in a liquid culture medium. The culture medium may contain carbon sources, nitrogen sources, minerals, etc. necessary for growing the transformant. The carbon source may include  
10       glucose, dextrin, soluble starch, sucrose, etc. The nitrogen source may include organic or inorganic substances such as ammonium salts, nitrates, corn steep liquor, peptone, casein, meat extracts, bean-cakes, potato extracts, etc. Examples of the minerals may  
15       include calcium chloride, sodium dihydrogen phosphate, magnesium chloride, etc. It is further allowable to add yeasts, vitamins, growth-promoting factors, etc. It is desired that the culture medium is pH from about 5 to about 8.

20       The culture medium for *Escherichia* microorganism is preferably an M9 medium containing, for example, glucose and casamino acids (Miller, Journal of Experiments in Molecular Genetics), 431-433, Cold Spring Harbor Laboratory, New York, 1972. Depending on  
25       necessity, the medium may be supplemented with drugs such as 3  $\beta$ -indolyl acrylic acid in order to improve efficiency of the promoter. In the case of an *Escherichia* host, the cultivation is carried out usually at about 15 to 43°C for about 3 to 24 hours. As  
30       required, aeration and stirring may be applied. In the case of *Bacillus* host, the cultivation is carried out usually at about 30 to 40°C for about 6 to 24 hours. As required, aeration and stirring may be also applied. In the case of the transformant in which the host is a  
35       yeast, the culture medium used may include, for example, a Burkholder minimum medium [Bostian, K.L. et al., Proc.

Natl. Acad. Sci. USA, Vol. 77, 4505 (1980)], an SD medium containing 0.5% casamino acids [Bitter, G.A. et al., Proc. Natl. Acad. Sci. USA, Vol. 81, 5330 (1984)], etc. It is preferable that the pH of the culture medium is adjusted to be from about 5 to about 8. The cultivation is carried out usually at about 20 to 35°C for about 24 to 72 hours. As required, aeration and stirring may be applied. In the case of the transformant in which the host is an insect, the culture medium used may include those obtained by suitably adding additives such as passivated (or immobilized) 10% bovine serum and the like to the Grace's insect medium (Grace, T.C.C., Nature, 195, 788 (1962)). It is preferable that the pH of the culture medium is adjusted to be about 6.2 to 6.4. The cultivation is usually carried out at about 27°C for about 3 to 5 days. As desired, aeration and stirring may be applied. In the case of the transformant in which the host is an animal cell, the culture medium used may include MEM medium [Science, Vol. 122, 501 (1952)], DMEM medium [Virology, Vol. 8, 396 (1959)], RPMI 1640 medium [Journal of the American Medical Association, Vol. 199, 519 (1967)], 199 medium [Proceedings of the Society of the Biological Medicine, Vol. 73, 1 (1950)], etc. which are containing, for example, about 5 to 20% of fetal calf serum. It is preferable that the pH is from about 6 to about 8. The cultivation is usually carried out at about 30 to 40°C for about 15 to 60 hours. As required, medium exchange, aeration and stirring may be applied.

Separation and purification of the polypeptide from the above-mentioned cultures can be carried out according to methods described herein below.

To extract polypeptide from the cultured microorganisms or cells, the microorganisms or cells are collected by known methods after the cultivation,

suspended in a suitable buffer solution, disrupted by ultrasonic waves, lysozyme and/or freezing and thawing, etc. and, then, a crude extract of the polypeptide or partial peptide is obtained by centrifugation or filtration. Other conventional extracting or isolating methods can be applied. The buffer solution may contain a protein-denaturing agent such as urea or guanidine hydrochloride or a surfactant such as Triton X-100 (registered trademark, hereinafter often referred to as "TM").

In the case where the polypeptide is secreted into culture medium, supernatant liquid is separated from the microorganisms or cells after the cultivation is finished and the resulting supernatant liquid is collected by widely known methods. The culture supernatant liquid and extract containing the polypeptide or partial peptide can be purified by a suitable combination of widely known methods for separation, isolation and purification. The widely known methods of separation, isolation and purification may include methods which utilizes solubility, such as salting out or sedimentation with solvents methods which utilizes primarily a difference in the molecular size or weight, such as dialysis, ultrafiltration, gel filtration and SDS-polyacrylamide gel electrophoresis, methods utilizing a difference in the electric charge, such as ion-exchange chromatography, methods utilizing specific affinity such as affinity chromatography, methods utilizing a difference in the hydrophobic property, such as reverse-phase high-performance liquid chromatography, and methods utilizing a difference in the isoelectric point such as isoelectric electrophoresis, or chromatofocusing, etc.

In cases where the polypeptide thus obtained is in a free form, the free protein can be converted into a salt thereof by known methods or method analogous

thereto. In case where the polypeptide thus obtained is in a salt form vice versa, the protein salt can be converted into a free form or into any other salt thereof by known methods or method analogous thereto.

5       The polypeptide produced by the transformant can be arbitrarily modified or a polypeptide can be partly removed therefrom, by the action of a suitable protein-modifying enzyme before or after the purification. The protein-modifying enzyme may include trypsin,  
10   chymotrypsin, arginyl endopeptidase, protein kinase, glycosidase, etc. The activity of the polypeptide thus formed can be measured by experimenting the coupling (or binding) with receptor or by enzyme immunoassays (enzyme linked immunoassays) using specific antibodies.

15       The ligand polypeptide of the present invention has prolactin secretion modulating activity, i.e. prolactin secretion promoting and/or inhibiting activities. Thus, as will be understood from the Examples presented hereinafter, the ligand polypeptide  
20   of the present invention has prolactin secretion promoting activity and, therefore, finds application as a drug for preventing and/or treating various diseases associated with prolactin hyposecretion. On the other hand, the ligand polypeptide of the invention has a  
25   high affinity for the receptor proteins and, therefore, when used in an increased dose, causes desensitization for prolactin secretion, thus exhibiting prolactin secretion inhibiting activity. In this sense, it can be used as a drug for preventing and/or treating  
30   various diseases associated with prolactin hypersecretion.

          Therefore, the ligand polypeptide of the invention can be used with advantage as a prolactin secretion-stimulating agent for the prevention and  
35   treatment of certain diseases associated with prolactin secretion, such as hypoovarianism, gonocyst cacogenesis,

menopausal syndrome, euthyroid hypometabolism. In addition, the ligand polypeptide of the invention can be used with advantage as a aphrodisiac.

On the other hand, the ligand polypeptide of the invention can be used with advantage as a prolactin secretion inhibitory agent in the prevention and treatment of certain diseases associated with prolactin secretion, such as pituitary adenomatosis, brain tumor, emmeniopathy, autoimmune disease, prolactinoma, infertility, impotence, amenorrhea, galactorrhea, acromegaly, Chiari-Frommel syndrome, Argonz-del Castilo syndrome, Forbes-Albright syndrome, lymphoma, Sheehan syndrome or dyszoospermia.

In addition, the ligand poltpeptide of the present invention is used as a contraceptives based on its prolactin secretion inhibitory activity.

In addition, the ligand polypeptide of the invention can be used as a test reagent for study of the prolactin secretory function or a veterinary drug for use as a lactogogue in mammalian farm animals such as bovine, goat, and swine, and is even expected to find application in the elaboration of useful substances in such farm mammals and harvesting of the substances secreted into their milk.

In addition, the ligand polypeptide of the present invention has a function of modulating placental function, and can be used as an agent for treating or preventing chriocarcinomia, hydatid mole, irruption mole, abortion, unthrifty fetus, abnormal saccharometabolism, abnormal lipidmetabolism or oxytocia.

When the ligand polypeptide of the present invention is used as a pharmaceutical composition as described above, it can be used by conventional methods. For example, it can be used orally in the form of tablets which may be sugar coated as necessary,

capsules, elixirs, microcapsules etc., or non-orally in the form of injectable preparations such as aseptic solutions and suspensions in water or other pharmaceutically acceptable liquids. These preparations  
5 can be produced by mixing the polypeptide with physiologically acceptable carriers, flavoring agents, excipients, vehicles, antiseptics, stabilizers, binders etc. in unit dosage forms required for generally accepted manners of pharmaceutical making. Active  
10 ingredient contents in these preparations are set so that an appropriate dose within the specified range is obtained.

Additives which can be mixed in tablets, capsules etc. include binders such as gelatin, corn starch, tragacanth and gum arabic, excipients such as  
15 crystalline cellulose, swelling agents such as corn starch, gelatin and alginic acid, lubricants such as magnesium stearate, sweetening agents such as sucrose, lactose and saccharin, and flavoring agents such as  
20 peppermint, akamono oil and cherry. When the unit dosage form is the capsule, the above-mentioned materials may further incorporate liquid carriers such as oils and fats. Sterile compositions for injection can be formulated by ordinary methods of pharmaceutical  
25 making such as by dissolving or suspending active ingredients, naturally occurring vegetable oils such as sesame oil and coconut oil, etc. in vehicles such as water for injection.

Aqueous liquids for injection include physiological  
30 saline and isotonic solutions containing glucose and other auxiliary agents, e.g., D-sorbitol, D-mannitol and sodium chloride, and may be used in combination with appropriate dissolution aids such as alcohols, e.g., ethanol, polyalcohols, e.g., propylene glycol and  
35 polyethylene glycol, nonionic surfactants, e.g., polysorbate 80 (TM) and HCO-50 etc. Oily liquids

include sesame oil and soybean oil, and may be used in combination with dissolution aids such as benzyl benzoate and benzyl alcohol. Furthermore the above-mentioned materials may also be formulated with buffers, e.g., phosphate buffer and sodium acetate buffer; soothing agents, e.g., benzalkonium chloride, procaine hydrochloride; stabilizers, e.g., human serum albumin, polyethylene glycol; preservatives, e.g., benzyl alcohol, phenol; antioxidants etc. Normally, an appropriate ampule is filled in with the thus prepared injectable liquid. Because the thus-obtained preparation is safe and of low toxicity, it can be administered to humans or warm-blooded mammals, e.g., mouse, rats, guinea pig, rabbits, chicken, sheep, pigs, bovines, cats, dogs, monkeys, baboons, chimpanzees, for instance.

The dose of said polypeptide is normally about 0.1-100 mg, preferably 1.0-50 mg, and more preferably 1.0-20 mg per day for a patient of euthyroid hypometabolism (weighing 60 kg) in oral administration, depending on symptoms etc. In non-oral administration, it is advantageous to administer the polypeptide in the form of injectable preparation at a daily dose of about 0.01-30 mg, preferably about 0.1-20 mg, and more preferably about 0.1-10 mg per administration by an intravenous injection for a patient of euthyroid hypometabolism (weighing 60 kg), depending on subject of administration, target organ, symptoms, method of administration etc. For other animal species, corresponding doses as converted per 60 kg weight can be administered.

The G protein-coupled receptor protein for the above ligand polypeptide of the present invention may be any of G protein-coupled receptor proteins derived from various tissues, e.g. hypophysis, pancreas, brain, kidney, liver, gonad, thyroid gland, gall bladder, bone

marrow, adrenal gland, skin, muscle, lung, alimentary canal, blood vessel, heart, etc. of human and other warm-blooded animals, e.g. guinea pig, rat, mouse, swine, sheep, bovine, monkey, etc.; and comprising an amino acid sequence of SEQ ID NO:19, 20, 21, 22 or 23, or a substantial equivalent thereto. Thus, the G protein-coupled receptor protein includes, in addition to proteins comprising the SEQ ID NO:19, 20, 21, 22 or 23, those proteins comprising amino acid sequences of about 90-99.9% homology to the amino acid sequence of SEQ ID NO:19, 20, 21, 22 or 23 and having qualitatively substantially equivalent activity to proteins comprising the amino acid sequence of SEQ ID NO:19, 20, 21, 22, or 23. The activities which these proteins possess may include ligand binding activity and signal transduction activity. The term "substantially equivalent" means that the nature of the ligand binding activity and the like is equivalent. Therefore, it is allowable that even differences among grades such as the strength of ligand binding activity and the molecular weight of receptor protein are present.

To be further specific, the G protein-coupled receptor proteins include human pituitary-derived G protein-coupled receptor proteins which comprises the amino acid sequence of SEQ ID NO:19 or/and SEQ ID NO:20, mouse pancreas-derived G protein-coupled receptor proteins which comprises the amino acid sequence of SEQ ID NO:22, and mouse pancreas-derived G protein-coupled receptor proteins which comprises the amino acid sequence of SEQ ID NO:23. As the human pituitary-derived G protein-coupled receptor proteins which comprises the amino acid sequence of SEQ ID NO:19 and/or SEQ ID NO:20 include the human pituitary-derived G protein-coupled receptor protein which comprises the amino acid sequence of SEQ ID NO:21. The G protein-coupled receptor proteins further include proteins



wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues are deleted from the amino acid sequence of SEQ ID NO:19, 20, 21, 22 or 23, proteins wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues are added to the amino acid sequence of SEQ ID NO:19, 20, 21, 22, or 23, the proteins wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues in the amino acid sequence of SEQ ID NO:19, 20, 21, 22, or 23 are substituted with one or more other amino acid residues.

Here, the protein which comprises an amino acid sequence of SEQ ID NO:21 or a substantial equivalent thereto contains the full-length of the amino acid sequence for human pituitary-derived G protein-coupled receptor protein. The protein which comprises an amino acid sequence of SEQ ID NO:19 or/and SEQ ID NO:20 or a substantial equivalent thereto may be a partial peptide of the protein which comprises an amino acid sequence of SEQ ID NO:21 or a substantial equivalent thereto. The protein which comprises an amino acid sequence of SEQ ID NO:22 or SEQ ID NO:23 or a substantial equivalent thereto is a G protein-coupled receptor protein which is derived from mouse pancreas but since its amino acid sequence is quite similar to the amino acid sequence of SEQ ID NO:19 or/and SEQ ID NO:20 (cf. Example 8, Fig. 13 in particular), the protein which comprises an amino acid sequence of SEQ ID NO:22 or 23 or a substantial equivalent thereto is also subsumed in the category of said partial peptide of the protein which comprises an amino acid sequence of SEQ ID NO:21 or a substantial equivalent thereto.

Thus, the above-mentioned protein comprising an amino acid sequence of SEQ ID NO:21 or a substantial equivalent thereto or a partial peptide of the protein or a salt thereof, which will be described below, includes the protein comprising an amino acid sequence

of SEQ ID NO:19, 20, 22, or 23 or a substantial equivalent thereto, or a salt thereof.

Furthermore, the G protein-coupled receptor protein includes the protein in which the N-terminal Met has been protected with a protective group, e.g. C<sub>1-6</sub> acyl such as formyl or acetyl, the protein in which the N-terminal side of Gln has been cleaved in vivo to form pyroglutamic acid, the protein in which the side chain of any relevant constituent amino acid has been protected with a suitable protective group, e.g. C<sub>1-6</sub> acyl such as formyl or acetyl, and the complex protein such as glycoproteins available upon attachment of sugar chains.

The salt of G protein-coupled receptor protein includes the same kinds of salts as mentioned for the ligand polypeptide.

The G protein-coupled receptor protein or a salt thereof or a partial peptide thereof can be produced from the tissues or cells of human or other warm-blooded animals by the per se known purification technology or, as described above, by culturing a transformant carrying a DNA coding for the G protein-coupled receptor protein. It can also be produced in accordance with the procedures for peptide synthesis which are described above. The procedures for peptide synthesis is described in WO96/05302 in detail.

A partial peptide of G protein-coupled receptor protein may include, for example, a fragment containing an extracellular portion of the G protein-coupled receptor protein, i.e. the site which is exposed outside the cell membranes. Examples of the partial peptide are fragments containing a region which is an extracellular area (hydrophilic region) as analyzed in a hydrophobic plotting analysis of the G protein-coupled receptor protein, such as shown in Fig. 3, Fig. 4, Fig. 8, Fig. 11, or Fig. 14. Furthermore, a

fragment which partly contains a hydrophobic region may also be used. While peptides which separately contains each domain may be used too, peptides which contains multiple domains at the same time will be used as well.

5       The salt of a partial peptide of G protein-coupled receptor protein may be the same one as mentioned for the salt of ligand polypeptide.

10       The DNA coding for the G protein-coupled receptor protein may be any DNA comprising a nucleotide sequence encoding the G protein-coupled receptor protein which comprises an amino acid sequence of SEQ ID NO:19, 20, 21, 22, or 23 or a substantial equivalent thereto. It may also be any one of genomic DNA, genomic DNA library, tissue- or cell-derived cDNA, tissue- or cell-derived  
15       cDNA library, and synthetic DNA. The vector for such a library may include bacteriophage, plasmid, cosmid, and phagimide. Furthermore, using an RNA fraction prepared from a tissue or cells, a direct amplification can be carried out by the RT-PCR method.

20       To be specific, the DNA encoding the human pituitary-derived G protein-coupled receptor protein which comprises the amino acid sequence of SEQ ID NO:19 include a DNA which comprises the nucleotide sequence of SEQ ID NO:24. The DNA encoding the human pituitary-  
25       derived G protein-coupled receptor protein which comprises the amino acid sequence of SEQ ID NO:20 include a DNA which comprises the nucleotide sequence of SEQ ID NO:25. The DNA encoding the human pituitary-derived G protein-coupled receptor protein which  
30       comprises the amino acid sequence of SEQ ID NO:21 include a DNA which comprises the nucleotide sequence of SEQ ID NO:26. The DNA encoding the mouse pancreas-derived G protein-coupled receptor protein which  
35       comprises the amino acid sequence of SEQ ID NO:22 include a DNA which comprises the nucleotide sequence of SEQ ID NO:27. The DNA encoding the mouse pancreas-

derived G protein-coupled receptor protein which comprises the amino acid sequence of SEQ ID NO:23 include a DNA comprising the nucleotide sequence of SEQ ID NO:28.

5       A method for cloning the DNA completely coding for the G protein-coupled receptor protein, vector, promoter, host cell, a method for transformation, a method for culturing the transformant or a method for separation and purification of the G protein-coupled  
10       receptor protein may include the same one as mentioned for the ligand polypeptide.

      To be specific, the plasmid phGR3 obtained in Example 5, described hereinafter, is digested with the restriction enzyme SalI and the translation frame for  
15       the full-length cDNA encoding hGR3 is isolated. This frame is subjected to ligation to, for example, the expression vector pAKKO-111 for animal cell use which has been treated with BAP (bacterial alkaline phosphatase) after SalI digestion for inhibition of  
20       autocyclization. After completion of the ligation reaction, a portion of the reaction mixture is used for transfection of, for example, Escherichia coli DH5. Among the transformants obtained, a transformant in which the cDNA coding for hGR3 has been inserted in the  
25       forward direction with respect to a promoter, such as SR  $\alpha$ , which has been inserted into the expression vector beforehand is selected by mapping after cleavage with restriction enzymes or by nucleotide sequencing and the plasmid DNA is prepared on a production scale.

30       The thus-constructed DNA of the expression vector is introduced into CHO dhfr<sup>-</sup> cells using a kit for introducing a gene into animal cells by the calcium phosphate method, the liposome method or the like to provide a high G protein-coupled receptor protein  
35       (hGR3) expression CHO cell line.

      The resulting CHO cells are cultured in a nucleic

acid-free screening medium in a CO<sub>2</sub> incubator at 37°C using 5% CO<sub>2</sub> for 1-4 days so as to give the G protein-coupled receptor protein (hGR3).

5 The G protein-coupled receptor protein is purified from the above CHO cells using an affinity column prepared by conjugating an antibody to the G protein-coupled receptor protein or a partial peptide thereof to a support or an affinity column prepared by conjugating a ligand for the G protein-coupled receptor protein.

10 The activity of the G protein-coupled receptor protein thus formed can be measured by experimenting the binding with a ligand or by enzyme immunoassays using specific antibodies.

15 Hereinafter, a method for determining a ligand to the G protein-coupled receptor protein is described in detail.

20 The G protein-coupled receptor protein, the partial peptide thereof or a salt thereof is useful as a reagent for investigating or determining a ligand to said G protein-coupled receptor protein.

25 According to the present invention, methods for determining a ligand to the G protein-coupled receptor protein which comprises contacting the G protein-coupled receptor protein or the partial peptide thereof with the compound to be tested, and measuring the binding amount, the cell stimulating activity, etc. of the test compound to the G protein-coupled receptor protein or the partial peptide thereof are provided.

30 The compound to be tested may include not only known ligands such as angiotensins, bombesins, canavanoids, cholecystokinins, glutamine, serotonin, melatonins, neuropeptides Y, opioids, purine, vasopressins, oxytocins, VIP (vasoactive intestinal and related peptides), somatostatins, dopamine, motilins, 35 amylin, bradykinins, CGRP (calcitonin gene related

peptides), leukotrienes, pancreastatins, prostaglandins, thromboxanes, adenosine, adrenaline,  $\alpha$  - and  $\beta$  - chemokines such as IL-8, GRO  $\alpha$ , GRO  $\beta$ , GRO  $\gamma$ , NAP-2, ENA-78, PF4, IP10, GCP-2, MCP-1, HC14, MCP-3, I-309, MIP1  $\alpha$ , MIP-1  $\beta$ , RANTES, etc.; endothelins, enterogastrins, histamine, neurotensins, TRH, pancreatic polypeptides, galanin, modified derivatives thereof, analogues thereof, family members thereof and the like but also tissue extracts, cell culture supernatants, etc. of human or warm-blooded animals such as mice, rats, swines, cattle, sheep and monkeys, etc. For example, said tissue extract, said cell culture supernatant, etc. is added to the G protein-coupled receptor protein for measurement of the cell stimulating activity, etc. and fractionated by relying on the measurements whereupon a single ligand can be finally determined and obtained.

In one specific embodiment of the present invention, said method for determining the ligand includes a method for determining whether a sample (including a compound or a salt thereof) is capable of stimulating a target cell which comprises binding said compound with the G protein-coupled receptor protein either in the presence of the G protein-coupled receptor protein, the partial peptide thereof or a salt thereof, or in a receptor binding assay system in which the expression system for the recombinant receptor protein is constructed and used; and measuring the receptor-mediated cell stimulating activity, etc. Examples of said cell stimulating activities that can be measured include promoting or inhibiting biological responses, e.g. liberation of arachidonic acid, liberation of acetylcholine, liberation of endocellular  $\text{Ca}^{2+}$ , production of endocellular cAMP, production of endocellular cGMP, production of inositol phosphate, changes in the cell membrane potential, phosphorylation

of endocellular protein, activation of c-fos, decrease in pH, etc, and preferably liberation of arachidonic acid. Examples of said compound or a salt thereof capable of stimulating the cell via binding with the G protein-coupled receptor protein include peptides, proteins, nonpeptidic compounds, synthetic compounds, fermented products, etc.

In more specific embodiments of the present invention, said methods for screening and identifying a ligand includes:

- 1) a method of screening for a ligand to a G protein-coupled receptor protein, which comprises contacting a labeled test compound with a G protein-coupled receptor protein or a salt thereof or its partial peptide or a salt thereof, and measuring the amount of the labeled test compound binding with said protein or salt thereof or with said partial peptide or salt thereof;
- 2) a method of screening for a ligand to a G protein-coupled receptor protein, which comprises contacting a labeled test compound with cells containing the G protein-coupled receptor protein or the membrane fraction of said cell, and measuring the amount of the labeled test compound binding with said cells or said membrane fraction;
- 3) a method of screening for a ligand to a G protein-coupled receptor protein, which comprises contacting a labeled test compound with the G protein-coupled receptor protein expressed on cell membranes by culturing transformants carrying the G protein-coupled receptor protein-encoding DNA and measuring the amount of the labeled test compound binding with said G protein-coupled receptor protein;
- 4) a method of screening for a ligand to a G protein-coupled receptor protein, which comprises contacting a test compound with cells containing the G protein-coupled receptor protein, and measuring the cell

stimulating activity, e.g. promoting or inhibiting activity on biological responses such as liberation of arachidonic acid, liberation of acetylcholine, liberation of endocellular  $Ca^{2+}$ , production of endocellular cAMP, production of endocellular cGMP, production of inositol phosphate, changes in the cell membrane potential, phosphorylation of endocellular protein, activation of c-fos, lowering in pH, etc. via the G protein-coupled receptor protein; and

5) a method of screening for a ligand to the G protein-coupled receptor protein, which comprises contacting a test compound with the G protein-coupled receptor protein expressed on the cell membrane by culturing transformants carrying the G protein-coupled receptor protein-encoding DNA, and measuring at least one cell stimulating activity, e.g., an activity for promoting or inhibiting physiological responses such as liberation of arachidonic acid, liberation of acetylcholine, liberation of endocellular  $Ca^{2+}$ , production of endocellular cAMP, production of endocellular cGMP, production of inositol phosphate, changes in the cell membrane potential, phosphorylation of endocellular protein, activation of c-fos, lowering in pH etc. via the G protein-coupled receptor protein.

Described below are specific illustrations of the method for screening and identifying ligands.

First, the G protein-coupled receptor protein used for the method for determining the ligand may include any material so far as it contains a G protein-coupled receptor protein, a partial peptide thereof or a salt thereof although it is preferable to express large amounts of the G protein-coupled receptor proteins in animal cells.

In the manufacture of the G protein-coupled receptor protein, the above-mentioned method can be used and carried out by expressing said protein



encoding DNA in mammalian cells or in insect cells. With respect to the DNA fragment coding for a particular region such as an extracellular epitope, the extracellular domains, etc., complementary DNA may be used although the method of expression is not limited thereto. For example, gene fragments or synthetic DNA may be used as well.

In order to introduce the G protein-coupled receptor protein-encoding DNA fragment into host animal cells and to express it efficiently, it is preferred that said DNA fragment is incorporated into the downstream side of polyhedron promoters derived from nuclear polyhedrosis virus belonging to baculovirus, promoters derived from SV40, promoters derived from retrovirus, metallothionein promoters, human heat shock promoters, cytomegalovirus promoters, SR  $\alpha$  promoters, etc. Examinations of the quantity and the quality of the expressed receptor can be carried out by methods per se known to those of skill in the art or methods similar thereto based upon the present disclosure. For example, they may be conducted by methods described in publications such as Nambi, P. et al: The Journal of Biochemical Society, vol.267, pages 19555-19559 (1992).

Accordingly, with respect to the determination of the ligand, the material containing a G protein-coupled receptor protein or partial peptide thereof may include products containing G protein-coupled receptor proteins which are purified by methods per se known to those of skill in the art or methods similar thereto, peptide fragments of said G protein-coupled receptor protein, cells containing said G protein-coupled receptor protein, membrane fractions of the cell containing said protein, etc.

When the G protein-coupled receptor protein-containing cell is used in the determining method of the ligand, said cell may be immobilized with binding

agents including glutaraldehyde, formalin, etc. The immobilization may be carried out by methods per se known to those of skill in the art or methods similar thereto.

5       The G protein-coupled receptor protein-containing cells are host cells which express the G protein-coupled receptor protein. Examples of said host cells are microorganisms such as *Escherichia coli*, *Bacillus subtilis*, yeasts, insect cells, animal cells, etc.

10       The cell membrane fraction is a cell membrane-rich fraction which is prepared by methods per se known to those of skill in the art or methods similar thereto after disruption of cells. Examples of cell disruption may include a method for squeezing cells using a  
15       Potter-Elvehjem homogenizer, a disruption by a Waring blender or a Polytron manufactured by Kinematica, a disruption by ultrasonic waves, a disruption via blowing out cells from small nozzles together with applying a pressure using a French press or the like,  
20       etc. In the fractionation of the cell membrane, a fractionation method by means of centrifugal force such as a fractional centrifugal separation and a density gradient centrifugal separation is mainly used. For example, disrupted cellular liquid is centrifuged at a  
25       low speed (500 rpm to 3,000 rpm) for a short period (usually, from about one to ten minutes), the supernatant liquid is further centrifuged at a high speed (15,000 rpm to 30,000 rpm) usually for 30 minutes to two hours and the resulting precipitate is used as a  
30       membrane fraction. Said membrane fraction contains a lot of the expressed G protein-coupled receptor protein and a lot of membrane components such as phospholipids and membrane proteins derived from the cells.

35       The amount of the G protein-coupled receptor protein in the membrane fraction cell containing said G protein-coupled receptor protein is preferably  $10^3$  to

10<sup>6</sup> molecules per cell or, more preferably, 10<sup>5</sup> to 10<sup>7</sup> molecules per cell. Incidentally, the greater the expressed amount, the higher the ligand binding activity (specific activity) per membrane fraction whereby the construction of a highly sensitive screening system becomes possible and, moreover, it permits measurement of a large amount of samples within the same lot.

In conducting the above-mentioned methods 1) to 3) wherein ligands capable of binding with the G protein-coupled receptor protein are determined, a suitable G protein-coupled receptor fraction and a labeled test compound are necessary. The G protein-coupled receptor fraction is preferably a naturally occurring (natural type) G protein-coupled receptor, a recombinant G protein-coupled receptor having the activity equivalent to that of the natural type. Here, the term "activity equivalent to" means the equivalent ligand binding activity, etc. as discussed above.

Suitable examples of the labeled test compound include above-mentioned compound to be tested which are labeled with [<sup>3</sup>H], [<sup>125</sup>I], [<sup>14</sup>C], [<sup>35</sup>S], etc.

Specifically, the determination of ligands capable of binding with G protein-coupled receptor proteins is carried out as follows:

First, cells or cell membrane fractions containing the G protein-coupled receptor protein are suspended in a buffer suitable for the assay to prepare the receptor sample for conducting the method of determining the ligand binding with the G protein-coupled receptor protein. The buffer may include any buffer such as Tris-HCl buffer or phosphate buffer with pH 4-10, preferably, pH 6-8, etc., as long as it does not inhibit the binding of the ligand with the receptor. In addition, surface-active agents such as CHAPS, Tween 80<sup>TM</sup> (Kao-Atlas, Japan), digitonin, deoxycholate, etc.

and various proteins such as bovine serum albumin(BSA), gelatin, milk derivatives, etc. may be added to the buffer with an object of decreasing the non-specific binding. Further, a protease inhibitor such as PMSF, leupeptin, E-64 (manufactured by Peptide Laboratory), pepstatin, etc. may be added with an object of inhibiting the decomposition of the receptor and the ligand by protease. A test compound labeled with a predetermined (or certain) amount (5,000 cpm to 500,000 cpm) of [<sup>3</sup>H], [<sup>125</sup>I], [<sup>14</sup>C], [<sup>35</sup>S], etc. coexists in 0.01 ml to 10 ml of said receptor solution. In order to know the non-specific binding amount (NSB), a reaction tube to which a great excessive amount of the unlabeled test compound is added is prepared as well. The reaction is carried out at 0-50°C, preferably at 4-37°C for 20 minutes to 24 hours, preferably 30 minutes to three hours. After the reaction, it is filtered through a glass fiber filter or the like, washed with a suitable amount of the same buffer and the radioactivity remaining in the glass fiber filter is measured by means of a liquid scintillation counter or a gamma-counter. The test compound in which the count (B - NSB) obtained by subtracting the non-specific binding amount (NSB) from the total binding amount (B) is more than 0 cpm is identified as a ligand to the G protein-coupled receptor protein.

In conducting the above-mentioned methods 4) to 5) wherein ligands capable of binding with the G protein-coupled receptor protein are determined, the cell stimulating activity, e.g. the liberation of arachidonic acid, the liberation of acetylcholine, endocellular Ca<sup>2+</sup> liberation, endocellular CAMP production, the production of inositol phosphate, changes in the cell membrane potential, the phosphorylation of endocellular protein, the activation of c-fos, lowering of pH, the activation of G protein,

cell promulgation, etc.; mediated by the G protein-coupled receptor protein may be measured by known methods or by the use of commercially available measuring kits. To be more specific, G protein-coupled receptor protein-containing cells are at first cultured in a multi-well plate or the like.

In conducting the determination of ligand, it is substituted with a fresh medium or a suitable buffer which does not show toxicity to the cells in advance of the experiment, and incubated under appropriate conditions and for sufficient time after adding a test compound, etc. thereto. Then, the cells are extracted or the supernatant liquid is recovered and the resulting product is determined by each of the methods. When it is difficult to identify the production of the substance, e.g. arachidonic acid, etc. which is to be an index for the cell stimulating activity due to the decomposing enzyme contained in the cell, an assay may be carried out by adding an inhibitor against said decomposing enzyme. With respect to an activity such as an inhibitory action against cAMP production, it may be detected as an inhibitory action against the production of the cells whose fundamental production is increased by forskolin or the like.

The kit used for the method of determining the ligand binding with the G protein-coupled receptor protein includes a G protein-coupled receptor protein or a partial peptide thereof, cells containing the G protein-coupled receptor protein, a membrane fraction from the cells containing the G protein-coupled receptor protein, etc.

Examples of the kit for determining the ligand are as follows:

1. Reagent for Determining the Ligand.
  - 1) Buffer for Measurement and Buffer for Washing.
- The buffering product wherein 0.05% of bovine serum

albumin (manufactured by Sigma) is added to Hanks' Balanced Salt Solution (manufactured by Gibco).

This product may be sterilized by filtration through a membrane filter with a  $0.45\mu\text{m}$  pore size, and stored at  $4^{\circ}\text{C}$  or may be formulated upon use.

2) G protein-coupled receptor Protein Sample.

CHO cells in which G protein-coupled receptor proteins are expressed are subcultured at the rate of  $5 \times 10^5$  cells/well in a 12-well plate and cultured at  $37^{\circ}\text{C}$  in a humidified 5%  $\text{CO}_2$ /95% air atmosphere for two days to prepare the sample.

3) Labeled Test Compound.

The compound which is labeled with commercially available [ $^3\text{H}$ ], [ $^{125}\text{I}$ ], [ $^{14}\text{C}$ ], [ $^{35}\text{S}$ ], etc. or labeled with a suitable method.

The product in a state of an aqueous solution is stored at  $4^{\circ}\text{C}$  or at  $-20^{\circ}\text{C}$  and, upon use, diluted to  $1\mu\text{M}$  with a buffer for the measurement. In the case of a test compound which is barely soluble in water, it may be dissolved in an organic solvent such as dimethylformamide, DMSO, methanol and the like.

4) Unlabeled Test Compound.

The same compound as the labeled one is prepared in a concentration of 100 to 1,000-fold concentrated state.

2. Method of Measurement

1) G protein-coupled receptor protein-expressing CHO cells cultured in a 12-well tissue culture plate are washed twice with 1 ml of buffer for the measurement and then  $490\mu\text{l}$  of buffer for the measurement is added to each well.

2) Five  $\mu\text{l}$  of the labeled test compound is added and the mixture is made to react at room temperature for one hour. For measuring the nonspecific binding amount,  $5\mu\text{l}$  of the unlabeled test compound is added.

3) The reaction solution is removed from each well, which is washed with 1 ml of a buffer for the

measurement three times. The labeled test compound which is binding with the cells is dissolved in 0.2N NaOH-1% SDS and mixed with 4 ml of a liquid scintillator A manufactured by WAKO Pure Chemical, Japan.

4) Radioactivity is measured using a liquid scintillation counter such as one manufactured by Beckmann.

Each SEQ ID NO set forth in the SEQUENCE LISTING of the specification refers to the following sequence:

[SEQ ID NO:1] is an entire amino acid sequence of the bovine pituitary-derived ligand polypeptide encoded by the cDNA included in pBOV3.

[SEQ ID NO:2] is an entire nucleotide sequence of the bovine pituitary-derived ligand polypeptide cDNA.

[SEQ ID NO:3] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide which was obtained by purification and analysis of N-terminal sequence for P-3 fraction. The amino acid sequence corresponds to 23rd to 51st positions of the amino acid sequence of SEQ ID NO:1. [SEQ ID NO:4] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide which was obtained by purification and analysis of N-terminal sequence for P-2 fraction. The amino acid sequence corresponds to 34th to 52nd positions of the amino acid sequence of SEQ ID NO:1.

[SEQ ID NO:5] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide. The amino acid sequence corresponds to 23rd to 53rd positions of the amino acid sequence of SEQ ID NO:1.

[SEQ ID NO:6] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide. The amino acid sequence corresponds to 23rd to 54th positions of the amino acid sequence of SEQ ID NO:1.

[SEQ ID NO:7] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide. The amino acid

sequence corresponds to 23rd to 55th positions of the amino acid sequence of SEQ ID NO:1.

[SEQ ID NO:8] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide. The amino acid  
5 sequence corresponds to 34th to 53rd positions of the amino acid sequence of SEQ ID NO:1.

[SEQ ID NO:9] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide. The amino acid  
10 sequence corresponds to 34th to 54th positions of the amino acid sequence of SEQ ID NO:1.

[SEQ ID NO:10] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide. The amino acid  
sequence corresponds to 34th to 55th positions of the amino acid sequence of SEQ ID NO:1.

15 [SEQ ID NO:11] is a nucleotide sequence of DNA coding for the bovine pituitary-derived ligand polypeptide (SEQ ID NO:3).

[SEQ ID NO:12] is a nucleotide sequence of DNA coding for the bovine pituitary-derived ligand polypeptide  
20 (SEQ ID NO:4).

[SEQ ID NO:13] is a nucleotide sequence of DNA coding for the bovine pituitary-derived ligand polypeptide  
(SEQ ID NO:5).

25 [SEQ ID NO:14] is a nucleotide sequence of DNA coding for the bovine pituitary-derived ligand polypeptide (SEQ ID NO:6).

[SEQ ID NO:15] is a nucleotide sequence of DNA coding for the bovine pituitary-derived ligand polypeptide  
(SEQ ID NO:7).

30 [SEQ ID NO:16] is a nucleotide sequence of DNA coding for the bovine pituitary-derived ligand polypeptide (SEQ ID NO:8).

[SEQ ID NO:17] is a nucleotide sequence of DNA coding for the bovine pituitary derived ligand polypeptide  
35 (SEQ ID NO:9).

[SEQ ID NO:18] is a nucleotide sequence of DNA coding



for the bovine pituitary-derived ligand polypeptide (SEQ ID NO:10).

[SEQ ID NO:19] is a partial amino acid sequence of the human pituitary-derived G protein-coupled receptor protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA fragment included in p19P2.

[SEQ ID NO:20] is a partial amino acid sequence of the human pituitary-derived G protein-coupled receptor protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA fragment include in p19P2.

[SEQ ID NO:21] is an entire amino acid sequence of the human pituitary-derived G protein-coupled receptor protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA include in phGR3.

[SEQ ID NO:22] is a partial amino acid sequence of the mouse pancreatic  $\beta$ -cell line, MIN6-derived G protein-coupled receptor protein encoded by the mouse pancreatic  $\beta$ -cell line, MIN6-derived G protein-coupled receptor protein cDNA fragment having a nucleotide sequence (SEQ ID NO:27), derived based upon the nucleotide sequences of the mouse pancreatic  $\beta$ -cell line, MIN6-derived G protein-coupled receptor protein cDNA fragments each included in pG3-2 and pG1-10.

[SEQ ID NO:23] is a partial amino acid sequence of the mouse pancreatic  $\beta$ -cell line, MIN6-derived G protein-coupled receptor protein encoded by p5S38.

[SEQ ID NO:24] is a nucleotide sequence of the human pituitary-derived G protein-coupled receptor protein cDNA fragment include in p19P2.

[SEQ ID NO:25] is a nucleotide sequence of the human pituitary-derived G protein-coupled receptor protein cDNA fragment include in p19P2.

[SEQ ID NO:26] is an entire nucleotide sequence of the human pituitary-derived G protein-coupled receptor

protein cDNA include in phGR3.

[SEQ ID NO:27] is a nucleotide sequence of the mouse pancreatic  $\beta$ -cell line, MIN6-derived G protein-coupled receptor protein cDNA, derived based upon the  
5 nucleotide sequences of the mouse pancreatic  $\beta$ -cell line, MIN6-derived G protein-coupled receptor protein cDNA fragments each included in pG3-2 and pG1-10.

[SEQ ID NO: 28] is a nucleotide sequence of the mouse pancreatic  $\beta$ -cell line, MIN6-derived G protein-coupled  
10 receptor protein cDNA include in p5S38.

[SEQ ID NO:29] is a synthetic DNA primer for screening of cDNA coding for the G protein-coupled receptor protein.

[SEQ ID NO:30] is a synthetic DNA primer for screening  
15 of cDNA coding for the G protein-coupled receptor protein.

[SEQ ID NO:31] is a synthetic DNA primer for screening of cDNA coding for the G protein-coupled receptor protein.

[SEQ ID NO:32] is a synthetic DNA primer for screening  
20 of cDNA coding for the G protein-coupled receptor protein.

[SEQ ID NO:33] is a synthetic DNA primer for screening  
25 of cDNA coding for the G protein-coupled receptor protein.

[SEQ ID NO:34] is a synthetic DNA primer for screening of cDNA coding for the G protein-coupled receptor protein.

[SEQ ID NO:35] is a synthetic DNA primer for screening  
30 of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by P5-1.

[SEQ ID NO:36] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by P3-1.

35 [SEQ ID NO:37] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand

polypeptide, wherein the primer is represented by P3-2.

[SEQ ID NO:38] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by PE.

5 [SEQ ID NO:39] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by PDN.

[SEQ ID NO:40] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by FB.

[SEQ ID NO:41] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by FC.

15 [SEQ ID NO:42] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by BOVF.

[SEQ ID NO:43] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by BOVR.

20 [SEQ ID NO:44] is an entire amino acid sequence of the bovine genome-derived ligand polypeptide.

[SEQ ID NO: 45] is an entire amino acid sequence of the rat type ligand polypeptide encoded by the cDNA included in pRAV3.

25 [SEQ ID NO:46] is an entire nucleotide sequence of the rat type ligand polypeptide cDNA.

[SEQ ID NO:47] is an amino acid sequence of the rat type ligand polypeptide. The amino acid sequence corresponds to 22nd to 52nd positions of the amino acid sequence of SEQ ID NO:45.

30 [SEQ ID NO:48] is an amino acid sequence of the rat type ligand polypeptide. The amino acid sequence corresponds to 22nd to 53rd positions of the amino acid sequence of SEQ ID NO:45.

35 [SEQ ID NO:49] is an amino acid sequence of the rat type ligand polypeptide. The amino acid sequence

corresponds to 22nd to 54th positions of the amino acid sequence of SEQ ID NO:45.

[SEQ ID NO:50] is an amino acid sequence of the rat type ligand polypeptide. The amino acid sequence  
5 corresponds to 33rd to 52nd positions of the amino acid sequence of SEQ ID NO:45.

[SEQ ID NO:51] is an amino acid sequence of the rat type ligand polypeptide. The amino acid sequence  
10 corresponds to 33rd to 53rd positions of the amino acid sequence of SEQ ID NO:45.

[SEQ ID NO:52] is an amino acid sequence of the rat type ligand polypeptide. The amino acid sequence  
corresponds to 33rd to 54th positions of the amino acid sequence of SEQ ID NO:45.

15 [SEQ ID NO:53] is a nucleotide sequence encoding for the rat type ligand polypeptide of SEQ ID NO:47.

[SEQ ID NO:54] is a nucleotide sequence encoding for the rat type ligand polypeptide of SEQ ID NO:48.

20 [SEQ ID NO:55] is a nucleotide sequence encoding for the rat type ligand polypeptide of SEQ ID NO:49.

[SEQ ID NO:56] is a nucleotide sequence encoding for the rat type ligand polypeptide of SEQ ID NO:50.

[SEQ ID NO:57] is a nucleotide sequence encoding for the rat type ligand polypeptide of SEQ ID NO:51.

25 [SEQ ID NO:58] is a nucleotide sequence encoding for the rat type ligand polypeptide of SEQ ID NO:52.

[SEQ ID NO:59] is an entire amino acid sequence of the human type ligand polypeptide encoded by the cDNA included in PHOB7.

30 [SEQ ID NO:60] is an entire nucleotide sequence of the human type ligand polypeptide cDNA.

[SEQ ID NO:61] is an amino acid sequence of the human type ligand polypeptide. The amino acid sequence  
35 corresponds to 23rd to 53rd positions of the amino acid sequence of SEQ ID NO:59.

[SEQ ID NO:62] is an amino acid sequence of the human

type ligand polypeptide. The amino acid sequence corresponds to 23rd to 54th positions of the amino acid sequence of SEQ ID NO.59.

5 [SEQ ID NO:63] is an amino acid sequence of the human type ligand polypeptide. The amino acid sequence corresponds to 23rd to 55th positions of the amino acid sequence of SEQ ID NO.59.

[SEQ ID NO:64] is an amino acid sequence of the human type ligand polypeptide. The amino acid sequence  
10 corresponds to 34th to 53rd positions of the amino acid sequence of SEQ ID NO.59.

[SEQ ID NO:65] is an amino acid sequence of the human type ligand polypeptide. The amino acid sequence  
15 corresponds to 34th to 54th positions of the amino acid sequence of SEQ ID NO.59.

[SEQ ID NO:66] is an amino acid sequence of the human type ligand polypeptide. The amino acid sequence  
20 corresponds to 34th to 55th positions of the amino acid sequence of SEQ ID NO.59.

[SEQ ID NO:67] is a nucleotide sequence encoding for the human type ligand polypeptide of SEQ ID NO:61.

[SEQ ID NO:68] is a nucleotide sequence encoding for the human type ligand polypeptide of SEQ ID NO:62.

25 [SEQ ID NO:69] is a nucleotide sequence encoding for the human type ligand polypeptide of SEQ ID NO:63.

[SEQ ID NO:70] is a nucleotide sequence encoding for the human type ligand polypeptide of SEQ ID NO:64.

[SEQ ID NO:71] is a nucleotide sequence encoding for the human type ligand polypeptide of SEQ ID NO:65.

30 [SEQ ID NO:72] is a nucleotide sequence encoding for the human type ligand polypeptide of SEQ ID NO:66.

[SEQ ID NO:73] is a partial amino acid sequence of the ligand polypeptide, wherein Xaa of the 10th position is Ala or Thr, Xaa of the 11th position is Gly or Ser and  
35 Xaa of the 21st position is H, Gly or GlyArg.

[SEQ ID NO:74] is a partial amino acid sequence of the

ligand polypeptide, wherein Xaa of the 3rd position is Ala or Thr, Xaa of the 5th position is Gln or Arg and Xaa of the 10th position is Ile or Thr.

5 [SEQ ID NO:75] is a synthetic DNA primer for screening of cDNA coding for the rat type ligand polypeptide, wherein the primer is represented by RA.

[SEQ ID NO:76] is a synthetic DNA primer for screening of cDNA coding for the rat type ligand polypeptide, wherein the primer is represented by RC.

10 [SEQ ID NO:77] is a synthetic DNA primer for screening of cDNA coding for the rat type ligand polypeptide, wherein the primer is represented by rF.

[SEQ ID NO:78] is a synthetic DNA primer for screening of cDNA coding for the rat type ligand polypeptide, wherein the primer is represented by rR.

[SEQ ID NO:79] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by R1.

20 [SEQ ID NO:80] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by R3.

[SEQ ID NO:81] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by R4.

25 [SEQ ID NO:82] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by HA.

[SEQ ID NO:83] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by HB.

30 [SEQ ID NO:84] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by HE.

[SEQ ID NO:85] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by HF.

[SEQ ID NO:86] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by 5H.

5 [SEQ ID NO:87] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by 3HN.

[SEQ ID NO:88] is a synthetic DNA primer for screening of cDNA coding for the rat type G protein-coupled receptor protein (UHR-1), wherein the primer is represented by rRECF.

[SEQ ID NO:89] is a synthetic DNA primer for screening of cDNA coding for the rat type G protein-coupled receptor protein (UHR-1), wherein the primer is represented by rRECR.

15 [SEQ ID NO:90] is a synthetic DNA which is used for amplification of G3PDH, UHR-1 and ligand, wherein the primer represented by r19F.

[SEQ ID NO:91] is a synthetic DNA which is used for amplification of G3PDH, UHR-1 and ligand, wherein the primer represented by r19R.

20 [SEQ ID NO:92] is a N-terminal peptide of the ligand polypeptide, which is used for antigen. (Peptide-I)

[SEQ ID NO:93] is a C-terminal peptide of the ligand polypeptide, which is used for antigen. (Peptide-II)

25 [SEQ ID NO:94] is a peptide of the central portion in ligand polypeptide, which is used for antigen. (Peptide-III)

[SEQ ID NO:95] is a synthetic DNA primer for screening of cDNA coding for rat type G protein-coupled receptor protein (UHR-1).

30 [SEQ ID NO:96] is a synthetic DNA primer for screening of cDNA coding for rat type G protein-coupled receptor protein (UHR-1).

[SEQ ID NO:97] is a synthetic DNA primer used in Example 48.

35 [SEQ ID NO:98] is a synthetic DNA primer used in

Example 48.

[SEQ ID NO:99] is a synthetic DNA probe used in Example 48.

5       The transformant *Escherichia coli*, designated INV $\alpha$   
F'/p19P2, which is obtained in the Example 2 mentioned  
herein below, is on deposit under the terms of the  
Budapest Treaty from August 9, 1994, with the National  
10       Institute of Bioscience and Human-Technology (NIBH),  
Agency of Industrial Science and Technology, Ministry  
of International Trade and Industry, Japan and has been  
assigned the Accession Number FERM BP-4776. It is also  
on deposit from August 22, 1994 with the Institute for  
Fermentation, Osaka, Japan (IFO) and has been assigned  
15       the Accession Number IFO 15739.

      The transformant *Escherichia coli*, designated INV $\alpha$   
F'/pG3-2, which is obtained in the Example 4 mentioned  
herein below, is on deposit under the terms of the  
Budapest Treaty from August 9, 1994, with NIBH and has  
20       been assigned the Accession Number FERM BP- 4775. It  
is also on deposit from August 22, 1994 with IFO and  
has been assigned the Accession Number IFO 15740.

      The transformant *Escherichia coli*, designated  
JM109/phGR3, which is obtained in the Example 5  
25       mentioned herein below, is on deposit under the terms  
of the Budapest Treaty from September 27, 1994, with  
NIBH and has been assigned the Accession Number FERM  
BP-4807. It is also on deposit from September 22, 1994  
with IFO and has been assigned the Accession Number IFO  
30       15748.

      The transformant *Escherichia coli*, designated  
JM109/p5S38, which is obtained in the Example 8  
mentioned herein below, is on deposit under the terms  
of the Budapest Treaty from October 27, 1994, with NIBH  
35       and has been assigned the Accession Number FERM BP-  
4856. It is also on deposit from October 25, 1994 with



IFO and has been assigned the Accession Number IFO 15754.

5 The transformant *Escherichia coli*, designated JM109/pBOV3, which is obtained in the Example 20 mentioned herein below, is on deposit under the terms of the Budapest Treaty from February 13, 1996, with NIBH and has been assigned the Accession Number FERM BP-5391. It is also on deposit from January 25, 1996 with IFO and has been assigned the Accession Number IFO  
10 15910.

15 The transformant *Escherichia coli*, designated JM109/pRAV3, which is obtained in the Example 29 mentioned herein below, is on deposit under the terms of the Budapest Treaty from September 12, 1996, with NIBH and has been assigned the Accession Number FERM BP-5665. It is also on deposit from September 3, 1996 with IFO and has been assigned the Accession Number IFO 16012.

20 The transformant *Escherichia coli*, designated JM109/pHOV7, which is obtained in the Example 32 mentioned herein below, is on deposit under the terms of the Budapest Treaty from September 12, 1996, with NIBH and has been assigned the Accession Number FERM BP-5666. It is also on deposit from September 5, 1996  
25 with IFO and has been assigned the Accession Number IFO 16013.

#### [Industrial Application]

30 The ligand polypeptide of the present invention has prolactin secretion modulating activity, i.e. prolactin secretion promoting and/or inhibiting activities. Thus, as will be understood from the Examples presented hereinafter, the ligand polypeptide of the present invention has prolactin secretion promoting activity  
35 and, therefore, finds application as a drug for preventing and/or treating various diseases associated

with prolactin hyposecretion. On the other hand, the ligand polypeptide of the invention has a high affinity for the receptor proteins and, therefore, when used in an increased dose, causes desensitization for prolactin secretion, thus exhibiting prolactin secretion inhibiting activity. In this sense, it can be used as a drug for preventing and/or treating various diseases associated with prolactin hypersecretion.

Therefore, the ligand polypeptide of the invention can be used with advantage as a prolactin secretion-stimulating agent for the prevention and treatment of certain diseases associated with prolactin secretion, such as hypoovarianism, gonocyst cacogenesis, menopausal syndrome, euthyroid hypometabolism. In addition, the ligand polypeptide of the invention can be used with advantage as a aphrodisiac.

On the other hand, the ligand polypeptide of the invention can be used with advantage as a prolactin secretion inhibitory agent in the prevention and treatment of certain diseases associated with prolactin secretion, such as pituitary adenomatosis, brain tumor, emmeniopathy, autoimmune disease, prolactinoma, infertility, impotence, amenorrhea, galactorrhea, acromegaly, Chiari-Frommel syndrome, Argonz-del Castilo syndrome, Forbes-Albright syndrome, lymphoma, Sheehan syndrome or dyszoospermia.

In addition, the ligand poltpeptide of the present invention is used as a contraceptives based on its prolactin secretion inhibitory activity.

In addition, the ligand polypeptide of the invention can be used as a test reagent for study of the prolactin secretory function or a veterinary drug for use as a lactogogue in mammalian farm animals such as bovine, goat, and swine, and is even expected to find application in the elaboration of useful substances in such farm mammals and harvesting of the

substances secreted into their milk.

In addition, the ligand polypeptide of the present invention has a function of modulating placental function, and can be used as an agent for treating or preventing choriocarcinoma, hydatid mole, irruption mole, abortion, unthrifty fetus, abnormal saccharometabolism, abnormal lipidmetabolism or oxytocia.

10 [Examples]

Described below are working examples of the present invention which are provided only for illustrative purposes, and not to limit the scope of the present invention. [Reference Example 1]

15 Preparation of Synthetic DNA Primer for Amplifying DNA Coding for G protein-coupled receptor Protein

A comparisons of deoxyribonucleotide sequences coding for the known amino acid sequences corresponding to or near the first membrane-spanning domain each of human-derived TRH receptor protein (HTRHR), human-derived RANTES receptor protein (L10918, HUMRANTES), human Burkitt's lymphoma-derived unknown ligand receptor protein (X68149, HSBLR1A), human-derived somatostatin receptor protein (L14856, HUMSOMAT), rat-derived  $\mu$ -opioid receptor protein (U02083, RNU02083), rat-derived  $\kappa$ -opioid receptor protein (U00442, U00442), human-derived neuromedin B receptor protein (M73482, HUMNMBR), human-derived muscarinic acetylcholine receptor protein (X15266, HSHM4), rat-derived adrenaline  $\alpha_1$ B receptor protein (L08609, RATAADRE01), human-derived somatostatin 3 receptor protein (M96738, HUMSSTR3X), human-derived  $C_a$  receptor protein (HUMC5AAR), human-derived unknown ligand receptor protein (HUMRDC1A), human-derived unknown ligand receptor protein (M84605, HUMOPIODRE) and rat-derived adrenaline  $\alpha_1$ B receptor protein (M91466, RATA2BAR) was

made. As a result, highly homologous regions or parts were found.

Further, a comparison of deoxynucleotide sequences coding for the known amino acid sequences corresponding to or near the sixth membrane-spanning domain each of  
5 mouse-derived unknown ligand receptor protein (M80481, MUSGIR), human-derived bombesin receptor protein (L08893, HUMBOMB3S), human-derived adenosine A2 receptor protein (S46950, S46950), mouse-derived  
10 unknown ligand receptor protein (D21061, MUSGPCR), mouse-derived TRH receptor protein (S43387, S43387), rat-derived neuromedin K receptor protein (J05189, RATNEURA), rat-derived adenosine A1 receptor protein (M69045, RATA1ARA), human-derived neurokinin A receptor  
15 protein (M57414, HUMNEKAR), rat-derived adenosine A3 receptor protein (M94152, DATADENREC), human-derived somatostatin 1 receptor protein (M81829, HUMSRI1A), human-derived neurokinin 3 receptor protein (S86390, S86371S4), rat-derived unknown ligand receptor protein  
20 (X61496, RNCGPCR), human-derived somatostatin 4 receptor protein (L07061, HUMSSTR4Z) and rat-derived GnRH receptor protein (M31670, RATGNRHA) was made. As a result, highly homologous regions or parts were found.

The aforementioned abbreviations in the parentheses  
25 are identifiers (reference numbers) which are indicated when GenBank/EMBL Data Bank is retrieved by using DNASIS Gene/Protein Sequencing Data Base (CD019, Hitachi Software Engineering, Japan) and are usually called "Accession Numbers" or "Entry Names". HTRHR is,  
30 however, the sequence as disclosed in Japanese Patent Publication No. 304797/1993 (EPA 638645).

Specifically, it was planned to incorporate mixed bases relying upon the base regions that were in  
35 agreement with cDNAs coding for a large number of receptor proteins in order to enhance base agreement of sequences with as many receptor cDNAs as possible even

in other regions. Based upon these sequences, the degenerate synthetic DNA having a nucleotide sequence represented by SEQ ID NO:29 or SEQ ID NO:30 which is complementary to the homologous nucleotide sequence were produced. [Synthetic DNAs] 5'-CGTGG (G or C) C (A or C) T (G or C) (G or C) TGGGCAAC (A, G, C or T) (C or T) CCTG-3' (SEQ ID NO:29) 5'-GT (A, G, C or T) G (A or T) (A or G) (A or G) GGCA (A, G, C or T) CCAGCAGA (G or T) GGCAAA-3' (SEQ ID NO:30)

The parentheses indicate the incorporation of a plurality of bases, leading to multiple oligonucleotides in the primer preparation. In other words, nucleotide residues in parentheses of the aforementioned DNAs were incorporated in the presence of a mixture of plural bases at the time of synthesis. [Example 1]

#### Amplification of Receptor cDNA by PCR Using Human Pituitary Gland-Derived cDNA

By using human pituitary gland-derived cDNA (QuickClone, CLONTECH Laboratories, Inc.) as a template, PCR amplification using the DNA primers synthesized in Reference Example 1 was carried out. The composition of the reaction solution consisted of the synthetic DNA primers (SEQ: 5' primer sequence and 3' primer sequence) each in an amount of 1  $\mu$ M, 1ng of the template cDNA, 0.25 mM dNTPs, 1  $\mu$ l of Taq DNA polymerase and a buffer attached to the enzyme kit, and the total amount of the reaction solution was made to be 100  $\mu$ l. The cycle for amplification including 95°C for 1 min., 55°C for 1 min. and 72°C for 1 min. was repeated 30 times by using a Thermal Cycler (Perkin-Elmer Co.). Prior to adding Taq DNA polymerase, the remaining reaction solution was mixed and was heated at 95°C for 5 minutes and at 65°C for 5 minutes. The amplified products were confirmed relying upon 1.2% agarose gel electrophoresis and ethidium bromide

staining.

[Example 2]

Subcloning of PCR Product into Plasmid Vector and  
Selection of Novel Receptor Candidate Clone via  
5 Decoding Nucleotide Sequence of Inserted cDNA Region

The PCR products were separated by using a 0.8%  
low-melting temperature agarose gel, the band parts  
were excised from the gel with a razor blade, and were  
heat-melted, extracted with phenol and precipitated in  
10 ethanol to recover DNAs. According to the protocol  
attached to a TA Cloning Kit (Invitrogen Co.), the  
recovered DNAs were subcloned into the plasmid vector,  
pCR<sup>TM</sup>II (TM represents registered trademark). The  
recombinant vectors were introduced into E. coli INV $\alpha$   
15 F' competent cells (Invitrogen Co.) to produce  
transformants. Then, transformant clones having a  
cDNA-inserted fragment were selected in an LB agar  
culture medium containing ampicillin and X-gal. Only  
transformant clones exhibiting white color were picked  
20 with a sterilized toothpick to obtain transformant  
Escherichia coli INV $\alpha$ F'/p19P2.

The individual clones were cultured overnight in  
an LB culture medium containing ampicillin and treated  
with an automatic plasmid extracting machine (Kurabo  
25 Co., Japan) to prepare plasmid DNAs. An aliquot of the  
DNA thus prepared was cut by EcoRI to confirm the size  
of the cDNA fragment that was inserted. An aliquot of  
the remaining DNA was further processed with RNase,  
extracted with phenol/chloroform, and precipitated in  
30 ethanol so as to be condensed. Sequencing was carried  
out by using a DyeDeoxy terminator cycle sequencing kit  
(ABI Co.), the DNAs were decoded by using a fluorescent  
automatic sequencer, and the data of the nucleotide  
sequences obtained were read by using DNASIS (Hitachi  
35 System Engineering Co., Japan). The underlined  
portions represent regions corresponding to the

synthetic primers.

Homology retrieval was carried out based upon the determined nucleotide sequences [SEQ ID NO:24 and 25 (Here, the determined nucleotide sequence is the  
5 nucleotide sequence which the underlined portion is deleted from the sequence of Figure 1 or Figure 2 respectively)].

As a result, it was learned that a novel G protein-coupled receptor protein was encoded by the cDNA  
10 fragment insert in the plasmid, p19P2, possessed by the transformant *Escherichia coli* INV  $\alpha$  F'/p19P2. To further confirm this fact, by using DNASIS (Hitachi System Engineering Co., Japan) the nucleotide sequences were converted into amino acid sequences [SEQ ID NO:19  
15 and 20], and homology retrieval was carried out in view of hydrophobicity plotting [Figures 3 and 4] and at the amino acid sequence level to find homology relative to neuro peptide Y receptor proteins [Figure 5].

[Example 3]

20 Preparation of Poly(A)<sup>+</sup>RNA Fraction from Mouse Pancreatic  $\beta$ -Cell Strain, MIN6 and Synthesis of cDNA

A total RNA was prepared from the mouse pancreatic  $\beta$ -cell strain, MIN6 (Jun-ichi Miyazaki et al., Endocrinology, Vol. 127, No. 1, p.126-132) according to  
25 the guanidine thiocyanate method (Kaplan B.B. et al., Biochem. J., 183, 181-184 (1979) and, then, poly(A)<sup>+</sup>RNA fractions were prepared with a mRNA purifying kit (Pharmacia Co.). Next, to 5  $\mu$ g of the poly(A)<sup>+</sup>RNA fraction was added a random DNA hexamer (BRL Co.) as a  
30 primer, and the resulting mixture was subjected to reaction with mouse Moloney Leukemia virus (MMLV) reverse transcriptase (BRL Co.) in the buffer attached to the MMLV reverse transcriptase kit to synthesize complementary DNAs. The reaction product was extracted  
35 with phenol/chloroform (1:1), precipitated in ethanol, and was then dissolved in 30  $\mu$ l of TE buffer (10 mM

Tris-HCl at pH8.0, 1 mM EDTA at pH8.0).

[Example 4]

Amplification of Receptor cDNA by PCR Using MIN6-Derived cDNA and Sequencing

5 By using, as a template, 5  $\mu$ l of cDNA prepared from the mouse pancreatic  $\beta$ -cell strain, MIN6 in the above Example 3, PCR amplification using the DNA primers synthesized in Reference Example 1 was carried out under the same condition as in Example 1. The  
10 resulting PCR product was subcloned into the plasmid vector, pCRTMII, in the same manner as in Example 2 to obtain a plasmid, pG3-2. The plasmid pG3-2 was transfected into E. coli INV $\alpha$ F' to obtain transformed Escherichia coli INV $\alpha$ F'/pG3-2.

15 By using, as a template, 5  $\mu$ l of the cDNA prepared from the mouse pancreatic  $\beta$ -cell strain, MIN6, PCR amplification using DNA primers as disclosed in Libert F. et al., "Science, 244:569-572, 1989", i.e., a degenerate synthetic primer represented by the  
20 following sequence: 5'-CTGTG (C or T) G (C or T) (G or C) AT (C or T) GCIIT (G or T) GA (C or T) (A or C) G (G or C) TAC-3'

(SEQ ID NO:31)

wherein I is inosine; and a degenerate synthetic primer  
25 represented by the following sequence: 5'-A (G or T) G (A or T) AG (A or T) AGGGCAGCCAGCAGAI (G or C) (A or G) (C or T) GAA-3' (SEQ ID NO:32) wherein I is inosine, was carried out under the same conditions as in Example 1. The resulting PCR  
30 product was subcloned into the plasmid vector, pCRTMII, in the same manner as described in Example 2 to obtain a plasmid, pG1-10.

The reaction for determining the nucleotide sequence (sequencing) was carried out with a DyeDeoxy  
35 terminator cycle sequencing kit (ABI Co.), the DNA was decoded with the fluorescent automatic sequencer (ABI



Co.), and the data of the nucleotide sequence obtained were analyzed with DNASIS (Hitachi System Engineering Co., Japan).

Figure 6 shows a mouse pancreatic  $\beta$ -cell strain MIN6-derived G protein-coupled receptor protein-encoding DNA (SEQ ID NO:27) and an amino acid sequence (SEQ ID NO:22) encoded by the isolated DNA based upon the nucleotide sequences of plasmids pG3-2 and pG1-10 which are possessed by the transformant Escherichia coli INV  $\alpha$  F'/pG3-2. The underlined portions represent regions corresponding to the synthetic primers.

Homology retrieval was carried out based upon the determined nucleotide sequence [Figure 6]. As a result, it was learned that a novel G protein-coupled receptor protein was encoded by the cDNA fragment obtained. To further confirm this fact, by using DNASIS (Hitachi System Engineering Co., Japan) the nucleotide sequence was converted into an amino acid sequence [Figure 6], hydrophobicity plotting was carried out to confirm the presence of six hydrophobic regions [Figure 8]. Upon comparing the amino acid sequence with that of p19P2 obtained in Example 2, furthermore, a high degree of homology was found as shown in [Figure 7]. As a result, it is strongly suggested that the G protein-coupled receptor proteins encoded by pG3-2 and pG1-10 recognize the same ligand as the G protein-coupled receptor protein encoded by p19P2 while the animal species from which the receptor proteins encoded by pG3-2 and pG1-10 are derived is different from that from which the receptor protein encoded by p19P2 is.

[Example 5]

Cloning of cDNA Comprising Whole Coding Regions  
for Receptor Protein from Human Pituitary Gland-  
Derived cDNA Library

The DNA library constructed by Clontech Co. wherein  $\lambda$  gt11 phage vector is used (CLONTECH Laboratories,

Inc.; CLH L1139b) was employed as a human pituitary gland-derived cDNA library. The human pituitary gland cDNA library ( $2 \times 10^6$  pfu (plaque forming units)) was mixed with E. coli Y1090- treated with magnesium sulfate, and incubated at  $37^\circ\text{C}$  for 15 minutes followed by addition of 0.5% agarose (Pharmacia Co.) LB. The E. coli was plated onto a 1.5% agar (Wako-Junyaku Co.) LB plate (containing  $50 \mu\text{g/ml}$  of ampicillin). A nitrocellulose filter was placed on the plate on which plaques were formed and the plaque was transferred onto the filter. The filter was denatured with an alkali and then heated at  $80^\circ\text{C}$  for 3 hours to fix DNAs.

The filter was incubated overnight at  $42^\circ\text{C}$  together with the probe mentioned herein below in a buffer containing 50% formamide, 5 x SSPE (20 x SSPE (pH 7.4) is 3 M NaCl, 0.2 M  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 25 mM EDTA), 5 X Denhardt's solution (Nippon Gene, Japan), 0.1% SDS and  $100 \mu\text{g/ml}$  of salmon sperm DNA for hybridization.

The probe used was obtained by cutting the DNA fragment inserted in the plasmid, p19P2, obtained in Example 2, with EcoRI, followed by recovery and labelling by incorporation of [ $^{32}\text{P}$ ]dCTP (Dupont Co.) with a random prime DNA labelling kit (Amasham Co.).

It was washed with 2 x SSC (20 x SSC is 3 M NaCl, 0.3 M sodium citrate), 0.1% SDS at  $55^\circ\text{C}$  for 1 hour and, then, subjected to an autoradiography at  $-80^\circ\text{C}$  to detect hybridized plaques.

In this screening, hybridization signals were recognized in three independent plaques. Each DNA was prepared from the three clones. The DNAs digested with EcoRI were subjected to an agarose electrophoresis and were analyzed by the southern blotting using the same probe as the one used in the screening. Hybridizing bands were identified at about 0.7kb, 0.8kb and 2.0kb, respectively. Among them, the DNA fragment corresponding to the band at about 2.0kb ( $\lambda$  hGR3) was

selected. The  $\lambda$  hGR3-derived EcoRI fragment with a hybridizable size was subcloned to the EcoRI site of the plasmid, pUC18, and E. coli JM109 was transformed with the plasmid to obtain transformant E. coli JM109/phGR3. A restriction enzyme map of the plasmid, phGR3, was prepared relying upon a restriction enzyme map deduced from the nucleotide sequence as shown in Example 2. As a result, it was learned that it carried a full-length receptor protein-encoding DNA which was predicted from the receptor protein-encoding DNA as shown in Example 2.

[Example 6]

Sequencing of Human Pituitary Gland-Derived Receptor Protein cDNA

Among the EcoRI fragments inserted in the plasmid, phGR3, obtained in the above Example 5, the from EcoRI to NheI nucleotide sequence with about 1330bp that is considered to be a receptor protein-coding region was sequenced. Concretely speaking, by utilizing restriction enzyme sites that exist in the EcoRI fragments, unnecessary parts were removed or necessary fragments were subcloned in order to prepare template plasmids for analyzing the nucleotide sequence.

The reaction for determining the nucleotide sequence (sequencing) was carried out with a DyeDeoxy terminator cycle sequencing kit (ABI Co.), the DNA was decoded with the fluorescent automatic sequencer (ABI Co.), and the data of the nucleotide sequence obtained were analyzed with DNASIS (Hitachi System Engineering Co., Japan).

Figure 9 shows a nucleotide sequence of from immediate after the EcoRI site up to the NheI site encoded by phGR3. The nucleotide sequence of the human pituitary gland-derived receptor protein-encoding DNA corresponds to the nucleotide sequence (SEQ ID NO:26) of from 118th to 1227th nucleotides [Figure 9]. An

amino acid sequence of the receptor protein that is encoded by the nucleotide sequence is shown in SEQ ID NO:21.

[Example 7]

5 Northern Hybridization with Human Pituitary Gland-Derived Receptor Protein-Encoding phGR3

Northern blotting was carried out in order to detect the expression of phGR3-encoded human pituitary gland-derived receptor proteins obtained in Example 5  
10 in the pituitary gland at a mRNA level. Human pituitary gland mRNA (2.5  $\mu$ g, Clontech Co.) was used as a template mRNA and the same as the probe used in Example 5 was used as a probe. Nylon membrane (Pall Biodyne, U.S.A.) was used as a filter for northern  
15 blotting and migration of the mRNA and adsorption (sucking) thereof with the blotting filter was carried out according to the method as disclosed in Molecular Cloning, Cold Spring Harbor Laboratory Press, 1989.

The hybridization was effected by incubating the  
20 above-mentioned filter and probe in a buffer containing 50% formamide, 5 x SSPE, 5 X Denhardt's solution, 0.1% SDS and 100  $\mu$ g/ml of salmon sperm DNA overnight at 42°C. The filter was washed with 0.1 x SSC, 0.1% SDS at 50°C and, after drying with air, was exposed to an  
25 X-ray film (XAR5, Kodak) for three days at -80°C. The results were as shown in Figure 10 from which it is considered that the receptor gene encoded by phGR3 is expressed in the human pituitary gland.

[Example 8]

30 Amplification of Receptor cDNA by PCR Using MIN6-Derived cDNA and Sequencing

By using, as a template, 5  $\mu$ l of cDNA prepared from the mouse pancreatic  $\beta$ -cell strain, MIN6 in Example 3, PCR amplification using the DNA primers  
35 synthesized in Example 4 as disclosed in Libert F. et al., "Science, 244:569-572, 1989", i.e., a synthetic

primer represented by the following sequence: 5'-CTGTG  
(C or T) G (C or T) (G or C) AT (C or T) GCIIT (G or T)  
GA (C or T) (A or C) G (G or C) TAC-3' (SEQ ID NO:31)  
wherein I is inosine; and a synthetic primer  
5 represented by the following sequence: 5'-A (G or T) G  
(A or T) AG (A or T) AGGGCAGCCAGCAGAI (G or C) (A or G)  
(C or T) GAA-3' (SEQ ID NO:32) wherein I is inosine,  
was carried out under the same conditions as in Example  
1. The resulting PCR product was subcloned to the  
10 plasmid vector, pCRTMII, in the same manner as in  
Example 2 to obtain a plasmid, p5S38. The plasmid  
p5S38 was transfected into E. coli JM109 to obtain  
transformant Escherichia coli JM109/p5S38.

The reaction for determining the nucleotide  
15 sequence (sequencing) was carried out with a DyeDeoxy  
terminator cycle sequencing kit (ABI Co.), the DNA was  
decoded with the fluorescent automatic sequencer (ABI  
Co.), and the data of the nucleotide sequence obtained  
were read with DNASIS (Hitachi System Engineering Co.,  
20 Japan).

Figure 12 shows a mouse pancreatic  $\beta$ -cell strain  
MIN6-derived G protein-coupled receptor protein-  
encoding DNA (SEQ ID NO:28) and an amino acid sequence  
(SEQ ID NO:23) encoded by the isolated DNA based upon  
25 the nucleotide sequence of plasmid, p5S38. The  
underlined portions represent regions corresponding to  
the synthetic primers.

Homology retrieval was carried out based upon the  
determined nucleotide sequence [Figure 12]. As a  
30 result, it was learned that a novel G protein-coupled  
receptor protein was encoded by the cDNA fragment  
obtained. To further confirm this fact, by using  
DNASIS (Hitachi System Engineering Co., Japan), the  
nucleotide sequence was converted into an amino acid  
35 sequence [Figure 12], and hydrophobicity plotting was  
carried out to confirm the presence of four hydrophobic

regions [Figure 14]. Upon comparing the amino acid sequence with those encoded by p19P2 obtained in Example 2 and encoded by pG3-2 obtained in Example 4, furthermore, a high degree of homology was found as shown in Figure 13. As a result, it is strongly suggested that the mouse pancreatic  $\beta$ -cell strain, MIN6-derived G protein-coupled receptor protein encoded by p5S38 recognizes the same ligand as the human pituitary gland-derived G protein-coupled receptor protein encoded by p19P2 does while the animal species from which the receptor protein encoded by p5S38 is derived is different from that from which the receptor protein encoded by p19P2 is. It is also strongly suggested that the mouse pancreatic  $\beta$ -cell strain, MIN6-derived G protein-coupled receptor protein encoded by p5S38 recognized the same ligand as the mouse pancreatic  $\beta$ -cell strain, MIN6-derived G protein-coupled receptor proteins encoded by pG3-2 and pG1-10 do and they are analogous receptor proteins one another (so-called "subtype").

[Example 9]

Preparation of CHO cells which express phGR3

The plasmid phGR3 (Example 5) containing a cDNA encoding the full-length amino acid sequence of human pituitary receptor protein was digested with the restriction enzyme Nco I and electrophoresed on agarose gel and a fragment of about 1kb was recovered. Both ends of the recovered fragment were blunted with a DNA blunting kit (Takara Shuzo Co., Japan) and, with the SalI linker added, treated with SalI and inserted into the SalI site of pUC119 to provide plasmid S10. Then, S10 was treated with SalI and SacII to prepare a fragment of about 700 bp (containing the N-terminal coding region). Then, a fragment of about 700 bp (containing the C-terminal coding region including initiation and termination codons) was cut out from

phGR3 with Sac II and Nhe I. These two fragments were added to the animal cell expression vector plasmid pAKKO-111H (the vector plasmid identical to the pAKKO1.11 H described in Biochim. Biophys. Acta, Hinuma, S., et al., 1219 251-259, 1994) and a ligation reaction was carried out to construct a full-length receptor protein expression plasmid pAKKO-19P2.

E. coli transfected with pAKKO-19P2 was cultured and the pAKKO-19P2 plasmid DNA was mass-produced using QUIAGEN Maxi. A 20  $\mu$ g portion of the plasmid DNA was dissolved in 1 ml of sterile PBS, and in a gene transfer vial (Wako Pure Chemical Ind.), the solution was vortexed well for liposome formation. This liposome, 125  $\mu$ l, was added to CHOdhfr<sup>-</sup> cells subcultured at  $1 \times 10^6$  per 10cm-dia. dish 24 hr before and placed in fresh medium immediately before addition and overnight culture was carried out. After a further one-day culture in fresh medium, the medium was changed to a screening medium and the incubation was further carried out for a day. For efficient screening of transformants, subculture was carried out at a low cell density and only the cells growing in the screening medium were selected to establish a full-length receptor protein expression CHO cell line CHO-19P2.

[Example 10]

Confirmation of the amount of expression of the full-length receptor protein in the CHO-19P2 cell line at the transcription level

Using FastTrack Kit (Invitrogen), CHO cells transfected with pAKKO-19P2 according to the kit manual and mock CHO cells were used to prepare poly(A)<sup>+</sup>RNA. Using 0.02  $\mu$ g of this poly(A)<sup>+</sup>RNA, a cDNA was synthesized by means of RNA PCR Kit (Takara Shuzo, Co., Japan). The kind of primer used was a random 9mer and the total volume of the reaction mixture was 40  $\mu$ l. As a negative control of cDNA synthesis, a reverse

transcriptase-free reaction mixture was also provided. First, the reaction mixture was incubated at 30°C for 10 minutes to conduct an amplification reaction to some extent. Then, it was incubated at 42°C for 30 minutes to let the reverse transcription reaction proceed. The  
5 enzyme was inactivated by heating at 99°C for 5 minutes and the reaction system was cooled at 5°C for 5 minutes.

After completion of the reverse transcription reaction, a portion of the reaction mixture was  
10 recovered and after dilution with distilled water, extraction was carried out with phenol/chloroform and further with diethyl ether. The extract was subjected to precipitation from ethanol and the precipitate was dissolved in a predetermined amount of distilled water  
15 for use as a cDNA sample. This cDNA solution and the plasmid DNA (pAKKO-19P2) were serially diluted and using primers specific to full-length receptor protein, PCR was carried out. The sequences of the primers prepared according to the base sequence of the coding  
20 region of the full-length receptor protein were CTGACTTATTTTCTGGGCTGCCGC (SEQ ID NO:33) for 5' end and AACACCGACACATAGACGGTGACC (SEQ ID NO:34) for 3' end.

The PCR reaction was carried out in a total volume of 100  $\mu$ l using 1  $\mu$ M each of the primers, 0.5  $\mu$ l of Taq  
25 DNA polymerase (Takara Shuzo Co., Japan), the reaction buffer and dNTPs accompanying the enzyme, and 10  $\mu$ l of template DNA (cDNA or plasmid solution). First the reaction mixture was heat-treated at 94°C for 2 minutes for sufficient denaturation of the template DNA and  
30 subjected to 25 cycles of 95°C x 30 seconds, 65°C x 30 seconds, and 72°C x 60 seconds. After completion of the reaction, 10  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis and the detection and quantitative comparison of amplification products were  
35 carried out. As a result, a PCR product of the size (400 bp) predictable from the sequence of the cDNA



coding for the full-length receptor protein was detected [Fig. 15]. In the lane of the PCR reaction mixture using the product of the reverse transcriptase-free transcription system as the template, no specific band was detected, thus extruding the possibility of its being a PCR product derived from the genomic DNA of CHO cells. Moreover, no specific band appeared in the lane of mock cells, either. Therefore, it was clear that the product was not derived from the mRNA initially expressed in CHO cells [Fig. 15].

[Example 11]

Detection of the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells in a rat whole brain extract

A crude peptide fraction was prepared from rat whole brain by the following procedure. The rat whole brain enucleated immediately after sacrifice was frozen in liquefied nitrogen and stored at  $-80^{\circ}\text{C}$ . The frozen rat whole brain, 20 g (the equivalent of 10 rats) was finely divided and boiled in 80 ml of distilled water for 10 minutes. After the boiled tissue was quenched on ice, 4.7 ml of acetic acid was added at a final concentration of 1.0 M and the mixture was homogenized using a Polytron (20,000 rpm, 6 min.). The homogenate was stirred overnight and then centrifuged (10,000 rpm, 20 min.) to separate the supernatant. The sediment was homogenized in 40 ml of 1.0 M acetic acid and centrifuged again to recover the supernatant. The supernatants were pooled, diluted in 3 volumes of acetone, allowed to stand on ice for 30 minutes, and centrifuged (10,000 rpm, 20 min.) to recover the supernatant. The recovered supernatant was evaporated to remove acetone. To the resulting acetone-free concentrate was added 2 volumes of 0.05% trifluoroacetic acid(TFA)/ $\text{H}_2\text{O}$  and the mixture was applied to a reversed-phase C18 column (Prep C<sup>18</sup> 125Å).

Millipore). After application of the supernatant, the column was washed with 0.05% TFA/H<sub>2</sub>O, and gradient elution was carried out with 10%, 20%, 30%, 40%, 50%, and 60% CH<sub>3</sub>CN/0.05%TFA/H<sub>2</sub>O. The fractions were  
5 respectively divided into 10 equal parts and lyophilized. The dried sample derived from one animal equivalent of rat whole brain was dissolved in 20  $\mu$ l of dimethyl sulfoxide (DMSO) and suspended in 1 ml of Hank's balanced saline solution (HBSS) supplemented  
10 with 0.05% bovine serum albumin (BSA) to provide a crude peptide fraction.

The full-length receptor protein-expressed CHO cells and mock CHO cells were seeded in a 24-well plate, 0.5 x 10<sup>5</sup> cells/well, and cultured for 24 hours. Then,  
15 [<sup>3</sup>H] arachidonic acid was added at a final concentration of 0.25  $\mu$  Ci/well. Sixteen (16) hours after addition of [<sup>3</sup>H] arachidonic acid, the cells were rinsed with 0.05% BSA-HBSS and the above-mentioned crude peptide fraction was added, 400  $\mu$  l/well. The  
20 mixture was incubated at 37°C for 30 minutes and a 300  $\mu$  l portion of the reaction mixture (400  $\mu$  l) was added to 4 ml of a scintillator and the amount of [<sup>3</sup>H] arachidonic acid metabolite released into the reaction mixture was determined with a scintillation counter.  
25 As a result, an arachidonic acid metabolite-releasing activity specific to the full-length receptor protein expressed CHO cells (CHO-19P2) was detected in the 30% CH<sub>3</sub>CN fraction of the eluate [Fig. 16].

[Example 12]

30 Detection of the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells in a bovine hypothalamus extract

A crude peptide fraction was prepared from 360 g (the equivalent of 1 animals) of bovine brain tissue  
35 including hypothalamus in the same manner as in Example 11. A dried peptide sample per 0.05 animal was

dissolved in 40  $\mu$ l of DMSO and suspended in 2 ml of 0.05% BSA-HBSS and the detection of arachidonic acid metabolite-releasing activity was attempted in the same manner as in Example 11. As a result, the activity to specifically promote release of arachidonic acid metabolites from the CHO-19P2 cell line was detected in the fraction eluted with 30%  $\text{CH}_3\text{CN}$  from a C18 column to which the crude bovine hypothalamus peptide fraction had been applied [Fig. 17].

[Example 13]

Preparation of the activity (peptide) to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells by purification from bovine hypothalamus

A typical process for harvesting the activity to specifically promote release of arachidonic acid metabolites from the CHO-19P2 cell line by purification from bovine hypothalamus is now described. A frozen bovine brain tissue specimen including hypothalamus, 4.0 kg (the equivalent of 80 animals) was ground and boiled in 8.0 L of distilled water for 20 minutes. After quenching on ice, 540 ml of acetic acid was added at a final concentration of 1.0 M and the mixture was homogenized using a Polytron (10,000 rpm, 12 min.). The homogenate was stirred overnight and then centrifuged (9,500 rpm, 20 min) to recover a supernatant. The sediment was suspended in 4.0 L of 1.0 M acetic acid and homogenized with the Polytron and centrifuged again to recover a further supernatant. The supernatants were pooled and TFA was added at a final concentration of 0.05%. The mixture was applied to reversed-phase C18 (Prep C18 125Å, 160 ml; Millipore) packed in a glass column. After addition, the column was washed with 320 ml of 0.05% TFA/ $\text{H}_2\text{O}$  and 3-gradient elution was carried out with 10%, 30%, and 50%  $\text{CH}_3\text{CN}$ /0.05% TFA/ $\text{H}_2\text{O}$ . To the 30%  $\text{CH}_3\text{CN}$ /0.05% TFA/ $\text{H}_2\text{O}$

fraction was added 2 volumes of 20 mM  $\text{CH}_3\text{COONH}_4/\text{H}_2\text{O}$  and the mixture was applied to the cation exchange column HiPrep CM-Sepharose FF (Pharmacia). After the column was washed with 20 mM  $\text{CH}_3\text{COONH}_4/10\% \text{CH}_3\text{CN}/\text{H}_2\text{O}$ , 4-  
5 gradient elution was carried out with 100 mM, 200 mM, 500 mM, and 1000 mM  $\text{CH}_3\text{COONH}_4/10\% \text{CH}_3\text{CN}/\text{H}_2\text{O}$ . In the 200 mM  $\text{CH}_3\text{COONH}_4$  fraction, activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 was detected. Therefore, this fraction was diluted  
10 with 3 volumes of acetone, centrifuged for deproteination, and concentrated in an evaporator. To the concentrated fraction was added TFA (final concentration 0.1%) and the mixture was adjusted to pH4 with acetic acid and applied to 3 ml of the reversed-  
15 phase column RESOURCE RPC (Pharmacia). Elution was carried out on a concentration gradient of 15%-30%  $\text{CH}_3\text{CN}$ . As a result, activity to specifically promote the release of arachidonic acid metabolites from the CHO-19P2 cell line was detected in the 19%-21%  $\text{CH}_3\text{CN}$   
20 fraction. The active fraction eluted from RESOURCE RPC was lyophilized, dissolved with DMSO, suspended in 50 mM MES pH 5.0/10%  $\text{CH}_3\text{CN}$ , and added to 1 ml of the cation exchange column RESOURCE S. Elution was carried out on a concentration gradient of 0 M-0.7 M NaCl. As  
25 a result, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was detected in the 0.32 M-0.46 M NaCl fraction. The active eluate from RESOURCE S was lyophilized, dissolved with DMSO, suspended in 0.1% TFA/ $\text{H}_2\text{O}$ , and  
30 added to reversed-phase column C18 218TP5415 (Vydac), and elution was carried out on a concentration gradient of 20%-30%  $\text{CH}_3\text{CN}$ . As a result, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was detected in the  
35 three fractions 22.5%, 23%, and 23.5%  $\text{CH}_3\text{CN}$  (these active fractions are designated as P-1, P-2, and P-3)

[Fig. 18]. Of the three active fractions, the 23.5% CH<sub>3</sub>CN fraction (P-3) was lyophilized, dissolved with DMSO, suspended in 0.1% TFA/H<sub>2</sub>O, and added to the reversed-phase column diphenyl 219TP5415 (Vydac), and elution was carried out on a gradient of 22%-25% CH<sub>3</sub>CN. As a result, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was converged by recovered in one elution peak obtained with 23% CH<sub>3</sub>CN [Fig. 19]. The peak activity fraction from the reverse-phased column diphenyl 219TP5415 was lyophilized, dissolved with DMSO, suspended in 0.1% TFA/H<sub>2</sub>O, and added to the reversed-phase column  $\mu$ RPC C2/C18 SC 2.1/10 (Pharmacia), and elution was carried out on a gradient of 22%-23.5% CH<sub>3</sub>CN. As a result, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was detected in the two peaks eluted with 23.0% and 23.2% CH<sub>3</sub>CN [Fig. 20].

[Example 14]

Determination of the amino acid sequence of the peptide having the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells as purified from bovine hypothalamus

The amino acid sequence of the peptide (P-3) having activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells as purified in Example 13 was determined. The fraction of peak activity from the reversed-phase  $\mu$ RPC C2/C18 SC 2.1/10 was lyophilized and dissolved in 20  $\mu$ l of 70% CH<sub>3</sub>CN and analyzed for amino acid sequence with the peptide sequencer (ABI.491). As a result, the sequence defined by SEQ ID NO:3 was obtained. However, the 7th and 19th amino acids were not determined by only the analysis of amino acid sequence.

[Example 15]

Preparation of the active substance (peptide)  
which specifically promotes release of arachidonic  
acid metabolites from CHO-19P2 cells as purified  
from bovine hypothalamus

5       Of the three active fractions obtained with Vydac  
C18 218TP5415 in Example 13, the active fraction (P-2)  
eluted with 23.0% CH<sub>3</sub>CN was further purified. This  
active fraction was lyophilized, dissolved with DMSO,  
suspended in 0.1% TFA/distilled H<sub>2</sub>O, and added to  
10       reversed-phase column diphenyl 219TP5415 (Vydac), and  
elution was carried out on a gradient of 21.0%-24.0%  
CH<sub>3</sub>CN. As a result, activity to specifically promote  
release of arachidonic acid metabolites from CHO-19P2  
cells was detected in a peak eluted with 21.9% CH<sub>3</sub>CN.  
15       This fraction was lyophilized, dissolved with DMSO,  
suspended in 0.1% TFA/distilled H<sub>2</sub>O, and added to  
reversed-phase  $\mu$ RPC C2/C18 SC 2.1/10 (Pharmacia), and  
elution was carried out on a CH<sub>3</sub>CN gradient of 21.5%-  
23.0%. As a result, the activity to specifically  
20       promote release of arachidonic acid metabolites from  
CHO-19P2 cells converged in one peak eluted with 22.0%  
CH<sub>3</sub>CN[Fig. 21].

[Example 16]

25       Determination of the amino acid sequence of the  
peptide (P-2) purified from bovine hypothalamus  
which specifically promotes release of arachidonic  
acid metabolites from CHO-19P2 cells

30       The amino acid sequence of the peptide (P-2) having  
the activity to specifically promote release of  
arachidonic acid metabolites from CHO-19P2 cells as  
purified in Example 15 was determined. The peak  
activity fraction from the reversed-phase column  $\mu$ RPC  
C2/C18 SC 2.1/10 was lyophilized, dissolved in 20  $\mu$ l of  
70% CH<sub>3</sub>CN, and analyzed for amino acid sequence with  
35       the peptide sequencer (ABI, 492) (SEQ ID NO:4).

[Example 17]

Preparation of a poly(A)<sup>+</sup>RNA fraction from bovine hypothalamus and synthesis of a cDNA

Using Isogen (Nippon Gene), total RNA was prepared from one animal equivalent of bovine hypothalamus. Then, using Fast Track (Invitrogen), a poly(A)<sup>+</sup>RNA fraction was prepared. From 1  $\mu$ g of this poly(A)<sup>+</sup>RNA fraction, cDNA was synthesized using 3' RACE system (GIBCO BRL) and Marathon cDNA amplification kit (Clontech) according to the manuals and dissolved in 20 and 10  $\mu$ l, respectively.  
[Example 18]

Acquisition of cDNA coding for the amino acid sequence established in Example 14

To obtain a cDNA coding for a polypeptide comprising the amino acid sequence established in Example 14, the acquisition of a base sequence coding for SEQ ID NO:1 was attempted in the first place. Thus, primers P5-1 (SEQ ID NO:35), P3-1 (SEQ ID NO:36), and P3-2 (SEQ ID NO:37) were synthesized. (In the Sequence Table, I represents inosine). Using 0.5  $\mu$ l of the cDNA prepared by 3' RACE in Example 17 as a template and EXTAq (Takara Shuzo Co., Japan) as DNA polymerase, 2.5  $\mu$ l of accompanying buffer, 200  $\mu$ M of accompanying dNTP, and primers P5-1 and P3-1 were added each at a final concentration of 200 nM, with water added to make 25  $\mu$ l, and after one minute at 94°C, the cycle of 98°C x 10 seconds, 50°C x 30 seconds, 68°C x 10 seconds was repeated 30 times. This reaction mixture was diluted 50-fold with tricine-EDTA buffer and using 2.5  $\mu$ l of the dilution as a template and the primer combination of P5-1 and P3-2, the reaction was carried out in otherwise the same manner as described above. As the thermal cycler, Gene Amp 9600 (Perkin Elmer) was used. The amplification product was subjected to 4% agarose electrophoresis and ethidium bromide staining and a band of about 70 bp was cut out and subjected to

thermal fusion, phenol extraction, and ethanol precipitation. The recovered DNA was subcloned into plasmid vector PCRTMII according to the manual of TA Cloning kit (Invitrogen). The vector was then  
5 introduced into E. coli JM109 and the resultant transformant was cultured in ampicillin-containing LB medium. The plasmid obtained with an automatic plasmid extractor (Kurabo) was reacted according to the manual of Dye Terminator Cycle Sequencing Kit (ABI) and  
10 decoded with a fluorescent automatic DNA sequencer (ABI). As a result, the sequence shown in Fig. 22 was obtained and confirmed to be part of the base sequence coding for SEQ ID NO:1.

[Example 19]

15 Acquisition of a bioactive polypeptide cDNA by RACE using the sequence established in Example 18  
First, for amplification (5' RACE) of the sequence at 5' end, the two primers PE (SEQ ID NO:38) and PDN (SEQ ID NO:39) were synthesized by utilizing the  
20 sequence shown in Fig. 22. The cDNA prepared using Marathon cDNA amplification kit in Example 17 was diluted 100-fold with tricine-EDTA buffer. Then, in the same manner as Example 2, a reaction mixture was prepared using 2.5  $\mu$ l of the dilution and a combination  
25 of the adapter primer AP1 accompanying the kit and the primer PE and after one minute at 94°C, the cycle of 98°C x 10 seconds and 68°C x 5 minutes was repeated 30 times. This reaction system was further diluted 50-fold with tricine-EDTA buffer and using 2.5  $\mu$ l of the  
30 dilution as a template and the changed primer combination of AP1 and PDN, the reaction was conducted at 94°C for one minute, followed by 4 cycles of 94°C x 1 minute, 98°C x 10 seconds, 72°C x 5 minutes, 4 cycles of 98°C x 10 seconds, 70°C x 5 minutes, and 26 cycles of  
35 98°C x 10 seconds, 68°C x 5 minutes. The amplification product was electrophoresed on 1.2% agarose gel and



stained with ethidium bromide and a band of about 150 bp was cut out and centrifugally filtered through a centrifugal filter tube (Millipore), extracted with phenol, and precipitated from ethanol. The recovered DNA was subcloned into plasmid vector PCR<sup>TM</sup>II according to the manual of TA Cloning Kit (Invitrogen). The vector was then introduced into E. coli JM109 and the resulting transformant was cultured and the sequence of the inserted cDNA fragment was analyzed as in Example 18. As a result, the sequence shown in Fig. 23 was obtained. Based on this sequence, primers FB (SEQ ID NO:40) and FG (SEQ ID NO:41) were synthesized and the 3' sequence was cloned (3' RACE). Using the same template as that for 5' RACE in the same quantity and the combination of the accompanying adapter primer AP1 with the primer FC, PCR was carried out at 94°C for 1 minute, followed by 5 cycles of 98°C x 10 seconds, 72°C x 5 minutes, 5 cycles of 98°C x 10 seconds, 70°C x 5 minutes, and 25 cycles of 98°C x 10 seconds, 68°C x 5 minutes. Then, using 2.5 µl of a 50- fold dilution of this reaction mixture in tricine-EDTA buffer as the template and the combination of the accompanying primer AP2 with the primer FB, the reaction was further conducted at 94°C for one minute, followed by 4 cycles of 98°C x 10 seconds, 72°C x 5 minutes, 4 cycles of 98°C x 10 seconds, 70°C x 5 minutes, and 27 cycles of 98°C x 10 seconds, 68°C x 5 minutes. The amplification product was electrophoresed on 1.2% agarose gel and stained with ethidium bromide and a band of about 400 bp was cut out and the DNA was recovered as in 5'-RACE. This DNA fragment was subcloned into plasmid vector pCRTMII and introduced into E. coli JM109 and the sequence of the inserted cDNA fragment in the resulting transformant was analyzed. From the results of 5' RACE and 3' RACE, the DNA sequence [Fig. 24] coding for the complete coding region of the bioactive polypeptide

defined by SEQ ID NO:1 was established. Thus, in Fig. 24 (a) and (b), the base134 is G, the base184 is T or C, and the base245 was T or C.

5 The cDNA shown in Fig. 24 was the cDNA encoding a polypeptide consisting of 98 amino acids. The fact that the amino acids in 1 - 22-positions comprise a cluster of hydrophobic amino acids taken together with the fact that the N-terminal region of the active peptide begins with Ser in 23-position as shown in  
10 Example 14 suggested that the amino acids 1-22 represent a secretion signal sequence. On the other hand, the Gly-Arg-Arg-Arg sequence in 54-57 positions of the polypeptide was found to be a typical amino acid sequence motif which exists in the event of cleavage of  
15 a bioactive peptide. As it is the case with this cleavage motif, it is known that because of the presence of Gly, the C-terminus of the product peptide is frequently amidated.

The P-3 N-terminal sequence data of Example 14 and  
20 P-2 N-terminal sequence data in Example 16 coupled with this GlyArgArgArg sequence suggest that at least some of the bioactive peptides cut out from the polypeptide encoded by this cDNA are defined by SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID  
25 NO:8, SEQ ID NO:9 or SEQ ID NO:10.

[Example 20]

Acquisition of a DNA fragment comprising the full coding region of bovine-derived bioactive polypeptide cDNA by PCR

30 Using the cDNA prepared with Marathon cDNA amplification kit in Example 17 as a template, a DNA fragment including the entire coding region of bioactive polypeptide cDNA was constructed. First, based on the sequence of cDNA elucidated in Example 19,  
35 two primers having base sequences defined by SEQ ID NO:42 and SEQ ID NO:43, respectively, were synthesized.

BOVF

5'-GTGTCGACGAATGAAGGCGGTGGGGCCTGGC-3' (SEQ ID NO:42)

BOVR (24 mer)

5'-AGGCTCCCGCTGTTATTCCTGGAC-3' (SEQ ID NO:43)

5 BOVF contains the initiation codon of bioactive polypeptide cDNA and is a sense sequence corresponding to -2 - +22 (A of the initiation codon ATG being reckoned as +1) with restriction enzyme SalI site added. On the other hand, BOVR is an antisense sequence corresponding to +285 - +309 which includes the termination codon of bioactive polypeptide cDNA.

10 The PCR was conducted as follows. The cDNA prepared using Marathon cDNA amplification kit in Example 17 was diluted 100-fold in tricine-EDTA buffer and using 2.5  $\mu$ l of the dilution, a reaction mixture was prepared as in Example 2 and subjected to 94°C x 1 minute, 3 cycles of 98°C x 10 seconds, 72°C x 5 minutes, 3 cycles of 98°C x 10 seconds, 70°C x 5 minutes, and 27 cycles of 98°C x 10 seconds, 68°C x 5 minutes. The amplification product was subjected to 2% agarose electrophoresis and ethidium bromide staining and a band of about 320 bp was cut out. The DNA was recovered and subcloned in plasmid vector pCRTMII as in Example 3. The vector was introduced into Escherichia coli JM109 to provide the transformant E. coli JM109/pBOV3. The sequence of the cDNA fragment inserted in the transformant was then analyzed. As a result, this DNA fragment was confirmed to be a fragment covering the entire coding region of the bioactive polypeptide cDNA.

30 [Example 21]

Synthesis of Ser-Arg-Ala-His-Gln-His-Ser-Met-Glu-Ile-Arg-Thr-Pro-Asp-Ile-Asn-Pro-Ala-Trp-Tyr-Ala-Gly-Arg-Gly-Ile-Arg-Pro-Val-Gly-Arg-Phe-NH<sub>2</sub> (19P2-L31)

35 1) Synthesis of Ser(Bzl)-Arg(Tos)-Ala-His(Bom)-Gln-

His(Bom)-Ser(Bzl)-Met-Glu(OcHex)-Ile-Arg(Tos)-Thr(Bzl)-  
Pro-Asp(OcHex)-Ile-Asn-Pro-Ala-Trp(CHO)-Tyr(Br-Z)-Ala-  
Gly-Arg(Tos)-Gly-Ile-Arg(Tos)-Pro-Val-Gly-Arg(Tos)-Phe-  
pMBHA-resin

- 5       The reactor of a peptide synthesizer (Applied Biosystems 430A) was charged with 0.71 g (0.5 mmole) of commercial p-methyl-BHA resin (Applied Biosystems, currently Perkin Elmer). After wetting with DCM, the initial amino acid Boc-Phe was activated by the
- 10       HOBt/DCC method and introduced into the p-methyl-BHA resin. The resin was treated with 50% TFA/DCM to remove Boc and make the amino group free and neutralized with DIEA. To this amino group was condensed the next amino acid Boc-Arg (Tos) by the
- 15       HOBt/DCC method. After the absence of unreacted amino function was verified by ninhydrin test, a sequential condensation of Boc-Gly, Boc-Val, Boc-Pro, Boc-Arg(Tos), Boc-Ile, Boc-Gly, Boc-Arg(Tos), Boc-Gly, Boc-Ala, Boc-Tyr(Br-Z) was carried out. The Boc-Ala, Boc-
- 20       Tyr (Br-Z), the condensation of which was found insufficient by ninhydrin test, was recondensed to complete the reaction. The resin was dried and a half of the resin was withdrawn. To the remainder, Boc-Trp(CHO), Boc-Ala, Boc-Pro, Boc-Asn, Boc-Ile, Boc-
- 25       Asp(OcHex), Boc-Pro, Boc-Thr(Bzl), Boc-Arg(Tos), Boc-Ile, Boc-Glu(OcHex), Boc-Met, Boc-Ser(Bzl), Boc-His(Bom), Boc-Gln, Boc-His(Bom), Boc-Ala, Boc-Arg(Tos), Boc-Ser(Bzl) were serially condensed and recondensed until sufficient condensation was confirmed by
- 30       ninhydrin test. After introduction of the full sequence of amino acids of 19P2-L31, the resin was treated with 50% TFA/DCM to remove Boc groups on the resin and, then, dried to provide 1.28 g of the peptide resin.
- 35       2) Synthesis of Ser-Arg-Ala-His-Gln-His-Ser-Met-Glu-Ile-Arg-Thr-Pro-Asp-Ile-Asn-Pro-Ala-Trp-Tyr-Ala-Gly-

Arg-Gly-Ile-Arg-Pro-Val-Gly-Arg-Phe-NH<sub>2</sub>(19P2-L31)

In a Teflon hydrogen fluoride reactor, the resin obtained in 1) was reacted with 3.8 g of p-cresol, 1 ml of 1,4-butanedithiol, and 10 ml of hydrogen fluoride at 0°C for 60 minutes. The hydrogen fluoride and 1,4-butanedithiol (1 ml) were distilled off under reduced pressure and the residue was diluted with 100 ml of diethyl ether, stirred, filtered through a glass filter, and the fraction on the filter was dried. This fraction was suspended in 50 ml of 50% acetic acid/H<sub>2</sub>O and stirred to extract the peptide. After separation of the resin, the extract was concentrated under reduced pressure to about 5 ml and chromatographed on Sephadex G-25 (2 x 90 cm). Development was carried out with 50% acetic acid/ H<sub>2</sub>O and the 114 ml - 181 ml fraction was pooled and lyophilized to recover 290 mg of white powders containing 19P2-L31. The powders were applied to a reversed-phase column of LiChroprep RP-18 (Merck) and repeatedly purified by gradient elution using 0.1% TFA/ H<sub>2</sub>O and 0.1% TFA-containing 30% acetonitrile/ H<sub>2</sub>O. The fraction eluted at about 25% acetonitrile was pooled and lyophilized to provide 71 mg of white powders.

Mass spectrum (M+H)<sup>+</sup> 3574.645

HPLC elution time 18.2 min.

Column conditions

Column: Wakosil 5C18 (4.6 x 100 mm)

Eluent: A (0.1% TFA/ H<sub>2</sub>O)

B (0.1% TFA-containing 50 %  
acetonitrile/ H<sub>2</sub>O)

Linear gradient elution from A to B (25 min.)

Flow rate: 1.0 ml/min.

[Example 22]

Synthesis of Ser-Arg-Ala-His-Gln-His-Ser-Met(O)-Glu-Ile-Arg-Thr-Pro-Asp-Ile-Asn-Pro-Ala-Trp-Tyr-Ala-Gly-Arg-Gly-Ile-Arg-Pro-Val-Gly-Arg-Phe-NH<sub>2</sub>(19P2-L31(O))

In 20 ml of 5% acetic acid/ H<sub>2</sub>O was dissolved 6 mg of synthetic 19P2-L31 and the Met only was selectively oxidized with 40  $\mu$ l of 30% H<sub>2</sub>O. After completion of the reaction, the reaction mixture was immediately  
5 applied to a reversed-phase column of LiChroprep RP-18 (Merck) for purification to provide 5.8 mg of the objective peptide.

Mass spectrum (M+H)<sup>+</sup> 3590.531

HPLC elution time 17.9 min.

10 Column conditions

Column: Wakosil 5C18 (4.6 x 100 mm)

Eluent: A (0.1% TFA/ H<sub>2</sub>O)

B (0.1% TFA-containing 50% acetonitrile/  
H<sub>2</sub>O)

15 Linear gradient elution from A to B (25 min.)

Flow rate: 1.0 ml/min.

[Example 23]

Synthesis of Thr-Pro-Asp-Ile-Asn-Pro-Ala-Trp-Tyr-Ala-Gly-Arg-Gly-Ile-Arg-Pro-Val-Gly-Arg-Phe-NH<sub>2</sub> (19P2-L20)

20 To the resin subjected to condensations up to Boc-Tyr(Br-Z) in Example 21-1) was further condensed Boc-Trp(CHO), Boc-Ala, Boc-Pro, Boc-Asn, Boc-Ile, Boc-Asp(OcHex), Boc-Pro, Boc-Thr(Bzl) serially in the same manner to provide 1.14 g of Boc-Thr(Bzl)-Pro-  
25 Asp(OcHex)-Ile-Asn-Pro-Ala-Trp(CHO)-Tyr(Br-Z)-Ala-Gly-Arg(Tos)-Gly-Ile-Arg(Tos)-Pro-Val-Gly-Arg(Tos)-Phe-pMBHA-resin. This resin was treated with hydrogen fluoride and columnwise purified in the same manner as  
Example 21-2) to provide 60 mg of white powders.

30 Mass spectrum (M+H)<sup>+</sup> 2242.149

HPLC elution time 10.4 min.

Column conditions

Column: Wakosil 5C18 (4.6 x 100 mm)

35 Eluent: A (0.1% TFA-containing 15% acetonitrile/  
H<sub>2</sub>O)

B (0.1% TFA-containing 45% aceto

nitrile/ H<sub>2</sub>O)

Linear gradient elution from A to B (15 min.)

Flow rate: 1.0 ml/min.

[Example 24]

5 Determination of arachidonic acid metabolites-releasing activity of synthetic peptide (19P2-L31)  
The activity of the peptide (19P2-L31) synthesized in Example 21 to specifically release arachidonic acid metabolites from CHO-19P2 cells was assayed in the same  
10 manner as Example 11. The synthetic peptide was dissolved in degassed distilled H<sub>2</sub>O at a concentration of 10<sup>-3</sup>M

and diluted with 0.05% BSA-HBSS and the activity to promote release of arachidonic acid metabolites from  
15 CHO-19P2 cells at each concentration was assayed using the amount of [<sup>3</sup>H]arachidonic acid metabolites as the indicator. As a result, concentration-dependent arachidonic acid metabolite-releasing activity was detected over the range of 10<sup>-12</sup>M - 10<sup>-6</sup>M [Fig. 25].

20 When the arachidonic acid metabolite-releasing activity of peptide 19P2-L31(O), i.e. the methionine-oxidation product of 19P2-L31 synthesized in Example 22, was compared with that of 19P2-L31, it was found that the activity of 19P2-L31(O) was equivalent to the activity  
25 of 19P2-L31 as can be seen from Fig. 26.

[Example 25]

Determination of arachidonic acid metabolites-releasing activity of synthetic peptide (19P2-L20)

30 The activity of the synthetic equivalent (19P2-L20) of natural peptide P-2 as synthesized in Example 23 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was determined as in Example 11. Thus, the synthetic peptide was dissolved in degassed distilled H<sub>2</sub>O at a final concentration of  
35 10<sup>-3</sup>M and this solution was serially diluted with 0.05% BAS-HBSS.

The activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells at each concentration was assayed using the amount of [<sup>3</sup>H]arachidonic acid metabolites as the indicator.

5       As a result, concentration-dependent arachidonic acid metabolite-releasing activity was detected over the range of  $10^{-12}$  -  $10^{-6}$ M in nearly the same degree as 19P2-L31 [Fig. 27].

[Example 26]

10       Analysis of the coding region base sequence of bovine genomic DNA

pBOV3 was digested with restriction enzyme EcoRI and after fractionation by agarose gel electrophoresis, the DNA corresponding to the cDNA fragment was recovered to prepare a probe. This DNA was labeled with <sup>32</sup>P using a multiprime DNA labeling kit (Amersham). About  $2.0 \times 10^6$  phages of Bovine Genomic Library (Clontech BL1015j) constructed using cloning vector EMBL3 SP6/T7 and Escherichia coli K802 as the host were seeded in an LB agar plate and cultured overnight for plaque formation. The plaques were transferred to a nitrocellulose filter and after alkaline modification and neutralization, heat-treated (80°C, 2 hours) to inactivate the DNA. This filter was incubated with the labeled probe in 50% formamide-Hybridization buffer (50% formamide, 5 x Denhardt solution, 4 x SSPE, 0.1 mg/ml heat-denatured salmon sperm DNA, 0.1% SDS) at 42°C overnight for hybridization. After this hybridization, the filter was washed with 2 x SSC, 0.1% SDS at room temperature for 1.5 hours, and further washed in the same buffer at 55°C for 30 minutes. Detection of the clone hybridizing with the probe was carried out on Kodak X-ray film (X-OMATMAR) after 4 days of exposure using a sensitization screen at -80°C. After development of the film, the film was collated with plate positions and the phages which had

15  
20  
25  
30  
35



hybridized were recovered. Then, plating and hybridization were repeated in the same manner for cloning of the phages.

The cloned phages were prepared on a large scale by the plate lysate method and the phage DNA was extracted. Then, cleavage at the restriction enzyme Sall and BamHI cleavage sites at both ends of the cloning site of the vector and detection of the inserted fragment derived from bovine genomic DNA was carried out by 1.2% agarose gel electrophoresis [Fig. 28]. As a result, in the case of BamHI digestion, 3 fragments were detected in addition to the bands derived from the phages. In the case of Sall digestion, one band overlapping the phage band was detected. The Sall-digested fragment being considered to harbor the full length and in order to subclone this fragment into a plasmid vector, it was ligated to BAP (E. coli-derived alkaline phosphatase)-treated plasmid vector pUC18 (Pharmacia) and introduced into E. coli JM109. From this microorganism, a genome-derived Sall fragment-inserted plasmid DNA was prepared on a production scale and the base sequence in the neighborhood of its coding region was analyzed using Perkin Elmer Applied Biosystems 370A fluorescent sequencer and the same manufacturer's kit. As a result, the sequence shown in Fig. 29 was obtained. Comparison with the coding region of cDNA reveals that because of its being derived from genomic DNA, the coding region is divided in two by a 472 bp intron [Fig. 30]. Fig. 31 and SEQ ID NO:44 present the amino acid sequence predicted from this bovine genome coding region (excluding the intron region).

[Example 27]

Preparation of rat medulla oblongata poly(A)<sup>+</sup> RNA fraction and synthesis of cDNA  
Using Isogen (Nippon Gene), total RNA was prepared

from the dorsal region of rat medulla oblongata and using FastTrack (Invitrogen), poly(A)<sup>+</sup>RNA fraction was prepared. To 5  $\mu$ g of this poly(A)<sup>+</sup>RNA was added the primer random DNA hexamer (BRL) and using Moloney mouse leukemia reverse transcriptase (BRL) and the accompanying buffer, complementary DNA was synthesized. The reaction product was precipitated from ethanol and dissolved in 12  $\mu$ l of DW. In addition, from 1  $\mu$ g of this poly(A)<sup>+</sup>RNA, a cDNA was synthesized using Marathon cDNA amplification kit (Clontech) according to the manual and dissolved in 10  $\mu$ l of DW.

[Example 28]

Acquisition of rat bioactive polypeptide cDNA by RACE

To obtain the full coding region of rat bioactive polypeptide cDNA, an experiment was performed in the same manner as the acquisition of bovine cDNA. First, PCR was carried out using the same primers P5-1 (SEQ ID NO:35) and P3-1 (SEQ ID NO:36) as used in Example 18 as primers and the complementary DNA synthesized in Example 27 using the primer random DNA hexamer (BRL) and Moloney mouse leukemia reverse transcriptase (BRL) as a template. The reaction system was composed of 1.25  $\mu$ l of the template cDNA, 200  $\mu$ M of dNTP, 1  $\mu$ M each of the primers, ExTaq (Takara Shuzo Co., Japan) as DNA polymerase, and 2.5  $\mu$ l of the accompanying buffer, with a sufficient amount of water to make a total of 25  $\mu$ l. The reaction was carried out at 94°C for 1 minute, followed by 40 cycles of 98°C x 10 seconds, 50°C x 30 seconds, and 72°C x 5 seconds, and the reaction mixture was then allowed to stand at 72°C for 20 seconds. The thermal cycler used was GeneAmp2400 (Perkin Elmer). The amplification product was subjected to 4% agarose electrophoresis and ethidium bromide staining and the band of about 80 bp was cut out. Then, in the manner described in Example 19, the DNA was recovered,

subcloned into plasmid vector pCRTMII, and introduced into E. coli JM109, and the inserted cDNA fragment was sequenced. As a result, a partial sequence of rat bioactive polypeptide could be obtained. Based on this sequence, two primers, namely RA (SEQ ID NO:75) for 3' RACE and RC (SEQ ID NO:76) for 5' RACE were synthesized and 5' and 3' RACEs were carried out.

RA:5'-CARCAYTCCATGGAGACAAGAACCCC-3'

(where R means A or G; Y means T or G) (SEQ ID NO:75)

RC:5'-TACCAGGCAGGATTGATACAGGGG-3'

(SEQ ID NO:76)

As a template, the template synthesized using Marathon cDNA amplification kit (Clontech) in Example 27 was diluted 40-fold with the accompanying tricine-EDTA buffer and 2.5  $\mu$ l of the dilution was used. As primers, RA and the adapter primer AP1 accompanying the kit were used for 3' RACE, and RC and AP1 for 5' RACE. The reaction mixture was prepared in the same manner as above. The reaction conditions were 94°C x 1 minute, 5 cycles of 98°C x 10 seconds, 72°C x 45 seconds, 3 cycles of 98°C x 10 seconds, 70°C x 45 seconds, and 40 cycles of 98°C x 10 seconds, 68°C x 45 seconds. As a result, a band of about 400 bp was obtained from 3' RACE and bands of about 400 bp and 250 bp from 5' RACE. These bands were recovered in the same manner as above and using them as templates and the primers used in the reaction, sequencing was carried out with Dye Terminator Cycle Sequencing Kit (ABI). As a result, the sequence up to poly A could be obtained from the region considered to be the 5' noncoding region.

[Example 29]

Acquisition of the full-length cDNA of rat bioactive polypeptide by PCR

Based on the sequence obtained in Example 28, two primers, viz. rF for the region including the

initiation codon (SEQ ID NO:77) and rR for the 3' side from the termination codon (SEQ ID NO:78), were synthesized to amplify the fragment including the full-length cDNA.

5' rF:5'-GGCATCATCCAGGAAGACGGAGCAT-3' (SEQ ID NO:77)  
rR:5'-AGCAGAGGAGAGGGAGGGTAGAGGA-3' (SEQ ID NO:78)

Using the cDNA prepared using Moloney mouse leukemia reverse transcriptase in Example 27 as a template and ExTaq (Takara Shuzo Co., Japan), PCR was carried out by repeating 40 cycles of 95°C x 30 seconds, 68°C x 60 seconds. The amplification product was subjected to agarose electrophoresis and ethidium bromide staining and a band of about 350 bp was cut out. The DNA was recovered, subcloned into plasmid vector pCRTMII, and introduced into E. coli JM109 as in Example 19. The plasmid was extracted from the transformant and the base sequence was determined. As a result, E. coli JM 109/pRAV3 having the full-length cDNA of rat bioactive polypeptide was obtained [Fig. 32].

[Example 30]

Synthesis of cDNA from the human total brain poly(A)<sup>+</sup>RNA fraction

From 1 µg of human total brain poly(A)<sup>+</sup>RNA fraction (Clontech), cDNA was synthesized with Marathon cDNA amplification kit (Clontech) according to the manual and dissolved in 10 µl. In addition, the random DNA hexamer (BRL) was added as primer to 5 µg of the same poly(A)<sup>+</sup>RNA fraction and using Moloney mouse leukemia reverse transcriptase (BRL) and the accompanying buffer, complementary DNA was synthesized. The reaction product was precipitated from ethanol and dissolved in 30 µl of TE.

[Example 31]

35 Acquisition of human bioactive polypeptide cDNA by RACE

From the amino acid sequence of rat bioactive polypeptide established in Example 28 [Fig. 33], the well-preserved regions of rat and bovine polypeptides were selected and the following 3 primers R1 (SEQ ID NO:79), R3 (SEQ ID NO:80), and R4 (SEQ ID NO:81) were synthesized. Then, amplification of the region flanked by them was attempted by PCR using human cDNA as a template. Referring to Fig. 33, bovine. aa represents the amino acid sequence of bovine polypeptide, bovine. seq represents the base sequence of the DNA coding for bovine polypeptide, and rat. seq represents the base sequence of the DNA coding for rat polypeptide.

R1:5'-ACGTGGCTTCTGTGCTTGCTGC-3' (SEQ ID NO:79)

R3:5'-GCCTGATCCCGCGGCCCGTGTACCA-3' (SEQ ID NO:80)

15 R4:5'-TTGCCCTTCTCCTGCCGAAGCGGCC-3' (SEQ ID NO:81)

The cDNA prepared using Marathon cDNA amplification kit (Clontech) in Example 30 was diluted 30-fold with tricine-EDTA buffer and 0.25  $\mu$ l of the dilution was used as a template. The reaction mixture was composed of 200  $\mu$ M of dNTP, 0.2  $\mu$ M each of the primers R1 and R4, a 50:50 mixture of Taq Start Antibody (Clontech) and DNA polymerase ExTaq (Takara Shuzo Co., Japan), 2.5  $\mu$ l of the accompanying buffer, and a sufficient amount of water to make a total of 25  $\mu$ l. The reaction conditions were 94°C x 1 minute, followed by 42 cycles of 98°C x 10 seconds, 68°C x 40 seconds, and 1 minute of standing at 72°C. Then, using 1  $\mu$ l of a 100-fold dilution of the above reaction mixture in tricine-EDTA buffer as a template, the same reaction mixture as above except that the primer combination was changed to R1 and R3 was prepared and PCR was carried out in the sequence of 94°C x 1 minute and 25 cycles of 98°C x 10 seconds, 68°C x 40 seconds. The amplification product was subjected to 4% agarose electrophoresis and ethidium bromide staining. As a result, a band of about 130 bp was obtained as

expected. This band was recovered in the same manner as in Example 28 and using the recovered fragment as a template, sequencing was carried out with Dye Terminator Cycle Sequencing Kit (ABI). As a result, a partial sequence of human bioactive polypeptide could be obtained. Therefore, based on this sequence, primers HA (SEQ ID NO:82) and HB (SEQ ID NO:83) were synthesized for 3' RACE and primers HE (SEQ ID NO:84) and HF (SEQ ID NO:85) for 5' RACE and 5' and 3' RACES were carried out.

HA:5'-GGCGGGGGCTGCAAGTCGTACCCATCG-3' (SEQ ID NO:82)  
HB:5'-CGGCACTCCATGGAGATCCGCACCCCT-3' (SEQ ID NO:83)  
HE:5'-CAGGCAGGATTGATGTTCAGGGGTGCGG-3' (SEQ ID NO:84)  
HF:5'-CATGGAGTGCCGATGGGTACGACTTGC-3' (SEQ ID NO:85)

As the template, 2.5  $\mu$ l of a 20-fold dilution of the cDNA prepared in Example 30 in tricine-EDTA buffer was used. For the initial PCR, reaction mixtures were prepared in the same manner as above except that HA and adapter primer AP1 were used for 3' RACE and HE and AP1 for 5' RACE. The reaction sequence was 94°C x 1 minute, 5 cycles of 98°C x 10 seconds, 72°C for 35 seconds, 5 cycles of 98°C x 10 seconds, 70°C x 35 seconds, and 40 cycles of 98°C x 10 seconds, 68°C x 35 seconds. Then, using 1  $\mu$ l of a 100-fold dilution of this reaction mixture in tricine-EDTA buffer as a template, a second PCR was carried out in the same cycles as the first PCR. However, the reaction mixture was prepared using primers HB and AP1 for 3' RACE or HF and AP2 for 5' RACE and Klen Taq (Clontech) as DNA polymerase and the accompanying buffer. As a result, a band of about 250 bp was obtained from 3' RACE and a band of about 150 bp from 5'-RACE. These bands were sequenced by the same procedure as above and using them in combination with the partial sequence obtained previously, the sequence from the region presumed to be 5'-noncoding region to polyA of human bioactive

polypeptide was obtained.

[Example 32]

Acquisition of human bioactive polypeptide full-length cDNA by PCR

5       Based on the sequence obtained in Example 31, two primers 5H (SEQ ID NO:86) and 3HN (SEQ ID NO:87) were synthesized for amplification of a fragment including full-length cDNA.

5H:5'-GGCCTCCTCGGAGGAGCCAAGGGATGA-3'       (SEQ ID NO:86)

10       3HN:5'-GGGAAAGGAGCCCGAAGGAGAGAGAG-3'       (SEQ ID NO:87)

Using 2.5  $\mu$ l of the cDNA prepared using Moloney mouse leukemia reverse transcriptase (BRL) in Example 30 as a template and the reaction mixture prepared using Klen Taq DNA polymerase (Clontech), the PCR  
15       reaction was conducted in the sequence of 94°C x 1 minute and 40 cycles of 98°C x 10 seconds, 68°C x 30 seconds. The fragment of about 360 bp obtained was recovered and subcloned (pCR™ 2.1 was used as the vector) in otherwise the same manner as Example 29.  
20       The plasmid was recovered and its base sequence was determined. As a result, E. coli JM109/pHOV7 harboring the human bioactive polypeptide full-length cDNA was obtained [Fig. 34]. In regard to the amino acid  
25       sequence of the translation region, a comparison was made between this human bioactive polypeptide and the bovine polypeptide shown in Example 20 or the rat polypeptide in Example 29 [Fig. 35].

[Example 33]

(1) Preparation of UHR-1 expression CHO cells

30       Recently, the orphan receptor UHR-1 has been cloned from the rat suprachiasmatic nucleus by Susan K. Welch and coworkers (Biochemical and Biophysical Research Communications, Vol. 209, No. 2, pp. 606-613, 1995).

35       Based on this report, the inventors of the present invention compared the amino acid sequence of

the protein encoded by the UHR-1 gene with the amino acid sequence of the protein encoded by hGR3.

As a result, the two sequences had 91.6% identity over 359 amino acids, suggesting that UHR-1 is a phGR3 homolog. In order to confirm that the protein encoded by UHR-1 functions as a receptor for 19P2-L31, the inventors of the present invention carried out a cloning of UHR-1 cDNA and subcloned it into CHO cells to construct a stable expression cell line as described below.

By the extraction using FastTrack™ Kit (Invitrogen), poly(A)<sup>+</sup>RNA was prepared from the anterior lobe of the rat hypophysis. Then, using 0.2  $\mu$ g of the poly(A)<sup>+</sup>RNA as a template, a cDNA was synthesized on a total reaction scale of 40  $\mu$ l using TaKaRa RNA PCR Kit (Takara Shuzo). The reaction product was extracted with phenol-chloroform (1:1), precipitated with ethanol, and dissolved in 10  $\mu$ l of distilled water. Based on the known nucleotide sequence of rat UHR-1 cDNA (GenBank, Accession Number S77867), the following two synthetic DNA primers were prepared.

(1) 5'-GTTACAG(GTCGAC)ATGACCTCAC-3'  
(SalI recognition sequence in parentheses) (SEQ ID NO:95)  
(2) 5'-CTCAGA(GCTAGC)AGAGTGTCATCAG-3'  
(NheI recognition sequence in parentheses) (SEQ ID NO:96)

Using the above pair of primers (1) and (2) and the cDNA synthesized by the procedure described above as the template, a PCR was carried out. For this reaction, 5  $\mu$ l of a 5-fold dilution of the cDNA solution, 1  $\mu$ l of a 1:1 mixture of Ex Taq (Takara Shuzo) and Taq Start Antibody (Clontech), 5  $\mu$ l of 10 x reaction buffer attached to Ex Taq, 4  $\mu$ l of dNTP, and 1  $\mu$ l each of the primers of 50  $\mu$ M concentration were



used and the whole amount was made up to 50  $\mu$ l with distilled water.

The PCR was performed according to the schedule of denaturing at 95 °C x 2 min. and 27 cycles each consisting of 95 °C x 30 sec., 65 °C x 30 sec. and 72 °C, 1 min., followed by final extension at 72 °C x 7 min. After completion of cycling, a portion of the reaction mixture was electrophoresed on an agarose gel. After ethidium bromide staining, a 1.1 kbp (approx.) band was excised, centrifugally filtered using a centrifugal filtration tube (Millipore), extracted with phenol, and precipitated from ethanol to recover the DNA. The recovered DNA was subcloned into the plasmid vector pCR™II according to the manual of TA Cloning Kit (Invitrogen) (pCRII-UHR-1) and introduced into Escherichia coli JM109. The resultant transformant was cultured in ampicillin-containing LB medium and the plasmid was extracted with an automatic plasmid extractor (Kurabo).

This plasmid was subjected to sequencing reaction using ABI PRISM Dye Terminator Cycle Sequencing Kit, FS (Perkin-Elmer) according to the manual and the nucleotide sequence was read out using a fluorescent automatated DNA sequencer (ABI).

The above sequencing revealed that the cDNA fragment obtained by PCR was a 1116bp fragment [Fig. 52]. Fig. 52 shows the nucleotide sequence of the full coding region of the rat UHR-1 constructed on the expression vector pAKKO-UHR-1 and the amino acid sequence encoded thereby. In Fig. 52, the underscored sequences (1) and (2) correspond to portions of the respective primer sequences. The bases different from those of the known nucleotide sequence (C in 664-position, G in 865-position, G in 897-position) are double-scored. The known nucleotide sequence presented here is a reproduction of GenBank Accession No. S77867.

One of those base substitutions involves an amino acid substitution of  $^{289}\text{Leu}$  (CTC)  $\rightarrow$   $^{289}\text{Val}$  (GTC). The construction of the UHR-1 expression vector was carried out as follows.

5 The pCRII-UHR-1 was cleaved with the restriction enzymes *NheI* (Takara Shuzo) and *SalI* (Takara Shuzo). The sample available on cleavage was electrophoresed on an agarose gel and stained with ethidium bromide, and the gel portion corresponding to the band was cut out.  
10 This gel fragment was put in a centrifuge tube with a filter (Millipore), frozen in a freezer, and thawed at room temperature. The tube was then centrifuged at 8000 rpm for 1 minute, whereupon a solution containing the DNA fragment was eluted out in the bottom of the  
15 filter. This solution was extracted with phenol, phenol-chloroform (1:1), and diethyl ether in the routine manner to remove impurities and the DNA was precipitated from ethanol to recover a cDNA fragment.

The pAKKO-111H was cleaved with the restriction  
20 enzymes *NheI* (Takara Shuzo) and *SalI* (Takara Shuzo) and the vector was isolated and extracted from an agarose gel in the same manner as above. Using Ligation System (Takara Shuzo), the cDNA fragment obtained above was reacted with the restriction enzyme digest of pAKKO-  
25 111H at 16°C for 30 minutes. Using a portion of this ligation product, Escherichia coli JM109 was transformed to construct a transformant, Escherichia coli JM109/pAKKO-UHR-1. This transformant was cultured overnight in 2 ml of ampicillin (50  $\mu\text{g/ml}$ )-containing  
30 LB medium and using an automatic plasmid extractor (Kurabo), the plasmid DNA (pAKKO-UHR-1) was obtained. The nucleotide sequence of the cDNA fragment-PAKKO-111H ligation site was analyzed with a fluorescent sequencer to confirm completion of the construction of the  
35 expression vector pAKKO-UHR-1.

(2) Introduction of the UHR-1 expression vector into

CHO dhfr<sup>r</sup> cells

In a 10 cm-diameter tissue culture dish,  $1 \times 10^6$  CHO dhfr<sup>r</sup> cells were seeded and cultured for 24 hours. From 20  $\mu$ g of the UHR-1 expression vector pAKKO-UHR-1 obtained in (1), a DNA-liposome complex was prepared using a liposome-mediated gene transfer kit (Gene Transfer, Nippon Gene). The medium was replaced with fresh one and the DNA-liposome complex was added and incubated overnight. The medium was replaced with fresh one again and further incubated for 1 day. After the medium was replaced with a transformant screening medium, the complex was incubated for 2 days. The cells were harvested from the dish by trypsin-EDTA treatment and recultured at a low cell density for an enhanced yield of the transformant. By the above procedure, a CHO-UHR-1 cell line capable of stable, high expression of UHR-1 could be cloned.

## [Example 34]

$^{125}$ I labeling of 19P2-L31 and a receptor-binding experiment using the labeled 19P2-L31

The radiolabeling of 19P2-L31 was carried out using [ $^{125}$ I]-Bolton-Hunter Reagent (NEN/DuPont; NEX-120). First, 200  $\mu$ l of [ $^{125}$ I]-Bolton-Hunter Reagent (2200 Ci/mmol) was transferred to a 500  $\mu$ l Eppendorf's tube and dried thoroughly with nitrogen gas. It was redissolved in 2  $\mu$ l of acetonitrile and, then, 4  $\mu$ l of 50 mM phosphate buffer (pH 8.0) and 4  $\mu$ l of  $3 \times 10^{-4}$  M synthetic 19P2-L31 were added. After mixing, the reaction was carried out at room temperature for 40 minutes. The reaction was then stopped with 5  $\mu$ l of 1.0 M glycine buffer and the whole reaction mixture was applied onto a reversed phased column (Tosoh; TSK gel ODS-80TMCTP) to separate [ $^{125}$ I]-labeled 19P2-L31 ([ $^{125}$ I]-19P2-L31). The fraction containing [ $^{125}$ I]-19P2-L31 was diluted with 2 volumes of 50 mM Tris-HCl (pH 7.5)-0.1% BSA-0.05% CHAPS, distributed in small aliquots, and

stored at  $-20^{\circ}\text{C}$ .

The receptor binding experiment was performed using CHO-19P2-9, CHO-UHR-1, and mock CHO as receptor expression CHO cells. CHO-19P2-9 cells were obtained by picking up a CHO-19P2 cell clone showing particularly high activity to stimulate the release of arachidonic acid metabolites by 19P2L-L31 from a limiting dilution culture in a 96-well microtiter plate. The mock CHO cells were control cells obtained by transformation with the expression vector pAKKO alone. Those cells, grown in tissue culture flasks, were respectively scraped off with 5 mM EDTA/PBS and resuspended in 0.05% BSA/0.05% CHAPS-containing HBSS at a density of  $0.5 \times 10^7$  cells/ml. To 100  $\mu\text{l}$  of this cell suspension was added [ $^{125}\text{I}$ ]-19P2-L31 at a final concentration of 200 pM. In addition, as an NSB (non-specific binding) experiment, 19P2-L31 was added to portions of the cell suspensions at a concentration of 200 nM. The reaction was performed at room temperature for 2.5 hours. After the reaction, B/F separation was carried out with a glass filter GF/F (Wattman) and the radioactivity trapped by the filter was counted with a gamma-counter as a receptor binding amount.

The results of receptor binding experiments using [ $^{125}\text{I}$ ]-19P2-L31 in living cells are shown in Fig. 36.

To 100  $\mu\text{l}$  of a cell suspension,  $0.5 \times 10^7$  cells/ml, was added [ $^{125}\text{I}$ ]-19P2-L31 at a final concentration of 200 pM, and after a 2.5-hour reaction at room temperature, the amount of [ $^{125}\text{I}$ ]-19P2-L31 bound to the receptor and the non-specific binding amount were determined with a gamma counter. The experiments were performed in triplicate and the mean values and standard deviations were calculated.

In the CHO cells in which hGR3 and UHR-1 were expressed, specific binding of [ $^{125}\text{I}$ ]-19P2-L31 was observed. Those results indicate that the protein

encoded by hGR3 or UHR-1 functions as a specific receptor of 19P2-L31.

[Example 35]

Specific stimulation of arachidonic acid metabolite release from CHO-19P2-9 and CHO-UHR1 by 19P2-L31

The action of 19P2-L31 to stimulate arachidonic acid metabolite release from CHO-19P2-9, CHO-UHR1, and mock CHO was assayed by the same procedure as described in Example 11.

Fig. 37 shows the results of assays of arachidonic acid metabolite releasing activity of 19P2-L31 in CHO-19P2-9 and CHO-UHR1. The experiments were performed in duplicate and the mean results are shown.

In CHO cells with expression of UHR1, too, a comparable degree of arachidonic acid metabolite releasing activity of 19P2-L31 was found as in CHO-19P2-9. Those results indicate that the protein encoded by UHR-1 functions as a specific receptor of 19P2-L31 as does hGR3.

[Example 36]

Assay of the expression of rat tissue ligand polypeptide and rat G protein-coupled receptor (UHR-1) by RT-PCR

(1) Preparation of poly(A)<sup>+</sup>RNA from rat tissues

Using an 8-week-old rat ( $\sigma^7$ ), poly(A)<sup>+</sup>RNAs from various tissues were prepared in amounts ranging from about 5 to about 30  $\mu$ g by the isolation of total RNA with Isogen (Nippon Gene) and subsequent purification with an oligo(dT)cellulose column (Pharmacia).

To completely remove the genome DNA from the poly(A)<sup>+</sup>RNA fraction, one unit of DNaseI (Gibco BRL, amplification grade) was used to decompose the DNA at room temperature. After addition of 25 mM EDTA, the reaction mixture was incubated at 65°C for 10 minutes to inactivate the DNaseI. The mixture was diluted to 40 ng/ $\mu$ l with water, and from a 160 ng portion thereof,

a cDNA was synthesized using 10 U AMV reverse transcriptase XL (Takara), 2.5  $\mu$ M random 9mer (Takara, final concentration 2.5  $\mu$ M), 10 mM Tris-HCl (pH 8.3), and 0.4 mM each dNTP. The synthetic reaction protocol was 30°C x 10 minutes followed by 42°C x 30 min, 99°C x 5 min, and 5°C x 5 min. The reaction product was precipitated from ethanol and dissolved in Tricine-EDTA buffer to give a total of 40  $\mu$ l (4 ng poly(A)<sup>+</sup>RNA/ $\mu$ l).  
(2) Construction of a positive control plasmid vector

Rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH, GenBank Accession No. M17701) was amplified by PCR using the cDNA synthesized from the rat pituitary GH3 poly(A)<sup>+</sup>RNA prepared using FastTrack Kit (Invitrogen) in the same manner as in (1) above as a template and Clontech's G3PDH amplification primer set. The UHR-1 was obtained by PCR using the cDNA of GH3, as a template, and the following primers, followed by subcloning into the pCR<sup>™</sup> 2.1 Vector of TA Cloning Kit (Invitrogen):

rRECF: 5'-CCTGCTGGCCATTCTCCTGTCTTAC-3' (SEQ ID NO:88)

rRECR: 5'-GGGTCCAGGTCCCGCAGAAGGTTGA-3' (SEQ ID NO:89)

Those were introduced into Escherichia coli JM109 to provide transformants. As the ligand peptide, JM109/pRAV3, already deposited, was used. After each of those transformants was cultured in ampicillin-containing LB medium, the plasmid was purified with Qiagen Plasmid Midi Kit (Qiagen) and, after the concentration was determined from optical density, used as a positive control plasmid vector.

### (3) RT-PCR

The cDNA solution and the positive control plasmid vector prepared in (1) and (2) above were used as templates, with or without dilution to a suitable concentration with water. For the amplification of G3PDH, UHR-1, and ligand peptide, Clontech's G3PDH Amplification Primer Set, rRECF/rRECR set, and the

following primer set were used, respectively, at a final concentration of 200 nM.

r19F: 5'-GAAGACGGAGCATGGCCCTGAAGAC-3' (SEQ ID NO:90)

r19R: 5'-GGCAGCTGAGTTGGCCAAGTCCAGT-3' (SEQ ID NO:91)

5 The reaction mixture consisted of 4  $\mu$ l of the diluted template, 200 nM each primers, dNTP (final concentration 100  $\mu$ M each), and KlenTaq (Clontech) as DNA polymerase and used after adjustment to 25  $\mu$ l with the buffer attached to KlenTaq and water. The  
10 amplification reaction conditions were as follows. G3PDH: 94°C x 1 min. followed by 26 cycles of 98°C x 10 sec, 65°C x 20 sec., and 72°C x 40 sec.; UHR-1 and ligand peptide: 94°C x 1 min, followed by 34 cycles of 98°C x 10 sec., 68°C x 25 sec. The amplification  
15 product was electrophoresed on an ethidium bromide-stained 1.2% or 4% agarose gel. The electrophoretogram was photographed by a CCD camera (Fotodyne, Foto/Ecrips) and the concentration of the band was digitalized and quantitated using an analytical  
20 software (Advanced American Biotechnology). The data for G3PDH was expressed in pg per 4 ng poly(A)<sup>+</sup>RNA and the data for UHR-1 and ligand peptide were expressed in pg per 4 ng poly(A)<sup>+</sup>RNA and, additionally, in the value found by dividing the pg value by pg for G3PDH [Figs.  
25 38 and 39].

As a result, UHR-1 and the ligand peptide were confirmed to be expressed in all tissues. The level of expression of UHR-1 was high in the hypophysis and a broad distribution was found in the brain, too, but the  
30 levels of expression in the peripheral tissues were not so high with the exception of the adrenal gland. On the other hand, the level of expression of the ligand peptide was high in the medula oblongata and hypothalamus, among brain tissues, and low in the  
35 hypophysis. In the peripheral tissues, the ligand peptide was expressed at comparatively high levels in

the lung, thymus, pancreas, kidney, adrenal, and testis. Those results suggest that UHR-1 and its ligand peptide are playing important roles in various tissues for the modulation of their functions.

5 [Example 37]

The influence of 19P2-L31 on glucose-induced increase in plasma insulin concentration

Wistar rats (8-10 weeks old, ♂) anesthetized with pentobarbital (65 mg/kg, i.p.) were transitorily  
10 dosed with glucose (86 mg/rat) alone or glucose in the same dose plus 19P2-L31 (675 pmol, 2.25 nmol, 6.75 nmol, or 67.5 nmol, per rat) via the common jugular vein, while the blood was serially drawn from the contralateral common jugular vein and the plasma  
15 insulin concentration was determined by radioimmunoassay. For this determination, Amersham's insulin assay kit was used.

19P2-L31 in a dose of 675 pmol, 2.25 nmol, or 6.75 nmol, suppressed the first-phase burst of plasma  
20 insulin concentration occurring 2 minutes following glucose loading and the second-phase moderate rise in plasma insulin concentration beginning around 6 minutes following administration. Administered in a dose of 67.5 nmol, 19P2-L31 completely inhibited both the  
25 first-phase and second-phase increases in insulin concentration [Fig. 40].

[Example 38]

The influence of the ligand polypeptide on the behaviors of mice

30 The inventors investigated the influence of 19P2-L31 and 19P2-L20 administered into a lateral ventricle of mice on their behaviors. Thus, mature male ICR mice (body weights at operation: ca 35 g) were anesthetized with pentobarbital 50 mg/kg i.p. and immobilized in a  
35 rat brain stereotaxic apparatus. The skull was exposed and a hole was drilled with a dental drill for



insertion of a guide cannula into one lateral ventricle. Thus, a stainless steel guide cannula (24G, 5 mm long) for intraventricular medication was inserted with its tip set at AP: +0.6 mm (from bregma), L: 1 mm (left), and H: -1 mm (from dura). The guide cannula was then rigidly secured to the skull with an adhesive. After operation, the mice were reared for at least 3 days for recuperation and then submitted to an experiment for behavioral analysis.

The spontaneous motor activity of mice was measured using a jiggle (spontaneous movement) cage made of clear acrylic resin, 24 x 37 x 30 cm, in a soundproof chamber. The mouse was individually housed in the above cage, and under a 12-hr light-and-dark cycle (ON: 6 to 18 o'clock) and with free access to water and food, the amount of spontaneous motor activity and the amount of rearing were respectively measured. The amount of spontaneous motor activity was measured with Supermex (Muromachi Machinery). The peptide or phosphate buffered saline (PBS) was administered at 2:30  $\pm$  30 min., p.m. For administration, a stainless steel microinjection cannula (30 G, 6 mm long) was passed through the guide cannula. The microinjection cannula was connected to a microsyringe pump via a Teflon tube and either PBS or a PBS solution of the peptide was infused at a flow rate of 2  $\mu$ l/min for 2 minutes. The microinjection cannula was left inserted for at least 2 minutes after completion of infusion and, then, removed and the amount of spontaneous motor activity was measured.

The results were expressed in mean  $\pm$  S.E.M. and the significance of the relative effect of the peptide and PBS treatments on motor activity was analyzed by Student's t-test. The difference at the 5% level of significance ( $p < 5\%$ ) on a two-tailed basis was regarded as being statistically significant. It is clear from

Fig. 41 that when 10 nmol of 19P2-L31 was administered, the spontaneous motor activity of mice was increased significantly during the period from 70 to 105 minutes after administration. The rearing behavior also showed a significant change in the like fashion. When, 1 nmol of 19P2-L31 was administered, no change was found in spontaneous activity and the amount of rearing was decreased significantly only at 105 minutes following administration [Fig. 42]. With 0.1 nmol of 19P2-31, the amount of spontaneous motor activity was increased significantly at 25, 40, and 70 minutes following administration. The amount of rearing also showed a similar trend but did not change significantly [Fig. 43]. With 0.01 nmol of 19P2-L31, spontaneous motor activity was increased significantly at 20 and 40 minutes following administration. The amount of rearing also showed a similar tendency toward increase but the change was not significant [Fig. 44].

[Example 39]

The influence of the ligand peptide on reserpine-induced hypothermia in mice

Mature male ICR mice (body weights at operation: ca 35 g) were anesthetized with pentobarbital 50 mg/kg i.p. and immobilized in a rat brain stereotaxic apparatus. The skull was exposed and a hole was drilled with a dental drill for indwelling a guide cannula in one lateral ventricle. A stainless steel guide cannula for intraventricular medication (24 G, 5 mm long) was inserted with its tip set at AP: +0.6 mm (from bregma), L: 1 mm (left), H: -1 mm (from dura). The guide cannula was rigidly secured to the skull with an adhesive. After operation, the mice were reared for at least 3 days for recuperation and the body temperature was then measured. Then, reserpine (Apopron Inj. 1 mg, Daiichi Pharmaceutical), 3 mg/kg, was injected subcutaneously, and 15 hours later the

mice were transferred to individual cages for body temperature measurement. A stainless steel microinjection cannula (30 G, 6 mm long) was passed into the guide cannula. The microinjection cannula was connected to a microinjection syringe pump via a Teflon tube and PBS or a PBS solution of the peptide was infused at a flow rate of 2  $\mu$ l/min. for 2 minutes. The microinjection cannula was left installed for at least 2 minutes following completion of infusion and, then, removed and the rectal temperature was measured.

The results were expressed in mean  $\pm$  S.E.M. and the significance of the relative effect of the peptide and PBS treatments on body temperature was analyzed by Student's t-test. The difference at the 5% level of significance on a two-tailed basis was regarded as being statistically significant. It is clear from Fig. 45 that when 10 nmol of 19P2-L31 was administered, the body temperature depressed by reserpine was elevated significantly as compared with the PBS control group. This elevation of body temperature peaked at 45 minutes following administration of 19P2-L31. On the other hand, no difference was found between the 19P2-L20 1 nmol group and the control group.

[Example 40]

The influence of the ligand polypeptide on rat blood pressure

The inventors of the present invention studied the influence of 19P2-L31 injected into the area postrema (AP) of medula oblongata on rat blood pressure. Mature male Wistar rats (body weights at operation: ca 300 g) were anesthetized with pentobarbital 50 mg/kg i.p. and immobilized in a rat brain stereotaxic apparatus. The incisal bar was set 3.3 mm below the interoral line. The skull was exposed and a hole was drilled with a dental drill for indwelling a guide cannula. In addition, anchor screws were embedded in 2

positions around the hole. A stainless steel guide cannula, AG-12 (inside dia. 0.4 mm, out. dia. 0.5 mm, Acom), was inserted with its tip situated in the superior domain of the area postrema. For this purpose, the guide cannula was inserted from the anterior direction at an angle of 20 degrees with the vertical direction (Fig. 46; the figure shows a microinjection cannula which is longer than the guide cannula by 1.0 mm). The stereotaxic coordinates of AP: -0.6 mm (from interoral line), L: 0.0 mm, H: +1.5 mm (from interoral line) were used with reference to the atlas of Paxinos and Watson (1986). The guide cannula was secured to the skull with an instant adhesive, a dental cement, and said anchor screws. In the guide cannula, a stainless steel dummy cannula, AD-12 (out. dia. 0.35 mm, Acom), was inserted and secured in position with a cap nut (Acom). Thereafter, the rats were reared in individual cages.

The animals were reared for about a week following cannulation for recuperation and a surgery was performed for measurement of conscious blood pressure. Thus, the rat was anesthetized with pentobarbital 50 mg/kg i.p. and immobilized in supine position on a dissection pad, and the left femoral artery was exposed. A polyethylene tube, SP35 (in. dia. 0.5 mm, out. dia. 0.9 mm, Natsume Seisakusho), was cut to about 60 cm in length and the cut tube was filled with 200 U/ml heparin-containing saline and inserted into the femoral artery over a distance of about 2.5 cm and secured in position. The other end of the tube was passed beneath the dorsal skin and exposed from the cervical (dorsal) region.

After one night following operation, the polyethylene tube was connected to a pressure transducer (Spectramed) and the blood pressure was measured. After the blood pressure reading had become steady, the

cap nut and dummy cannula were removed from the rat skull and, instead, a stainless steel microinjection cannula, AMI13 (in. dia. 0.17 mm, out. dia. 0.35 mm, Acom), connected to a Teflon tube (50 cm long, 0.1 mm in. dia., 0.4 mm out. dia., Acom), was inserted. The length of the microinjection cannula was adjusted beforehand so that its tip would be exposed from the guide cannula over a distance of 1 mm [Fig. 46]. The other end of the Teflon tube was connected to a microsyringe pump and 2  $\mu$ l of either PBS or a PBS solution of 19P2-L31 was injected into the area postrema at a flow rate of 1.0  $\mu$ l/min.

After blood pressure measurement, the microinjection cannula used for injection of 19P-L31 was removed and, instead, a microinjection cannula for infusion of a dye (Evans blue) was installed. The dye was similarly infused at a flow rate of 1.0  $\mu$ l/min for 2 minutes and after a waiting time of about 3 minutes the microinjection cannula was removed. The rat was decapitated and the brain was quickly enucleated and frozen. Using a cryostat, frozen sections were prepared and the infusion position of the dye was confirmed.

The above experiment revealed that the infusion of 10 nmol of 19P2-L31 into the area postrema caused a rise in blood pressure. A typical example of pulse wave and mean blood pressure is shown in Fig. 47.

[Example 41]

The influence of the ligand polypeptide on the plasma pituitary hormone level

The inventors of the present invention studied the influence of 19P2-L31 injected into the third ventricle on the plasma pituitary hormone levels. Mature male Wistar rats (body weights at operation: ca 290-350 g) were anesthetized with pentobarbital 50 mg/kg i.p. and each animal was immobilized in a rat

brain stereotaxic apparatus. The incisal bar was set 3.3 mm below the interoral line. The skull was exposed and using a dental bar a hole was drilled for indwelling a guide cannula. In addition, an anchor screw was embedded in one position around the hole. A stainless steel guide cannula, AG-12 (in. dia. 0.4 mm, out. dia. 0.5 mm, Acom), was inserted with its tip positioned in the superior domain of the third ventricle. The stereotaxic coordinates of AP: +7.2 mm (from interoral line), L: 0.0 mm, H: +2.0 mm (from interoral line) were used with reference to the atlas of Paxinos and Watson (1986). The guide cannula was rigidly secured to the skull with an instant adhesive, a dental cement, and said anchor screw. In the guide cannula, a stainless steel dummy cannula, AD-12 (out. dia. 0.35 mm, Acom) was passed and secured in position with a cap nut (Acom). After operation, the rats were reared in individual cages for at least 3 days for recuperation and then submitted to the experiment.

The rat operated on as above was anesthetized with pentobarbital 50 mg/kg i.p. and immobilized in supine position on a dissection pad. After the bilateral jugular veins were exposed, 400  $\mu$ l of blood was collected into a 1 ml tuberculin syringe with a 24 G needle (both from Terumo). To prevent clotting, the syringe was filled with 20  $\mu$ l of 200 U/ml heparin-containing saline ahead of time. The cap nut and dummy cannula were removed from the rat skull and, instead, a stainless steel microinjection cannula, AMI13 (in. dia. 0.17 mm, out. dia. 0.35 mm, Acom), connected to a Teflon tube (50 cm long, 0.1 mm in. dia, 0.4 mm out. dia. Acom) was inserted. The length of the microinjection cannula was adjusted beforehand so that its tip would be exposed from the guide cannula over a distance of 1 mm. The other end of the Teflon tube was connected to a microsyringe pump and 10  $\mu$ l of PBS or a

PBS solution of 19P2-L31 was injected into the third ventricle at a flow rate of  $2.5 \mu\text{l}/\text{min}$ . After a waiting time of 1 minute following completion of injection, the microinjection cannula was removed and the dummy cannula was reinstalled and secured with the cap nut. Immediately before intraventricular administration and 10, 20, 30, 40, and 60 minutes after the start of intraventricular administration,  $400 \mu\text{l}$  of blood was collected from the jugular vein. Each blood sample was centrifuged (5,000 rpm, 10 min.) using a high-speed refrigerated microcentrifuge (MR-150, Tomy precision Industry) and the supernatant (plasma) was recovered. The pituitary hormones [prolactin, luteinizing hormone (LH), adrenocorticotrophic hormone (ACTH), and thyrotropin (TSH), and growth hormone (GH)] in the plasma were respectively assayed by radioimmunoassays.

The results were expressed in mean  $\pm$  S.E.M. For the significance testing of the difference between the 19P2-L31/PBS group and the PBS group, Student's t-test was used. As a test for statistical significance, the 5% level was used. It can be seen from Fig. 48 that the plasmal level of growth hormone in the 19P2-L31 group was significantly decreased at 20 minutes after injection of 50 nmol into the third ventricle. The trend toward decrease was also observed at 10, 30, and 40 minutes as well but the changes were not significant. At 60 minutes after injection, there was no difference from the control group. The plasma prolactin, LH, ACTH, and TSH levels were not altered significantly

[Example 42]

Effects of ligand polypeptide on plasma growth hormone (GH) level in freely moving rats

Mature male Wistar rats were anesthetized with pentobarbital 50 mg/kg i.p. and, as in Example 41, a stainless-steel guide cannula AG-12 (0.4 mm in. dia.,

0.5 mm out. dia., EICOM) was implanted in position with its tip situated in the upper part of the third ventricle. After the operation the rats were housed in individual cages and kept for at least 3 days for recuperation and, then, a cannula (30 cm long, 0.5 mm in. dia., 0.9 mm out. dia., Natsume Seisakusho) filled in with heparin (200 U/ml)-containing saline was inserted into the right atrium from the right jugular vein under pentobarbital anesthesia. The rats were maintained overnight for complete arousal from anesthesia and then transferred to transparent acrylic cages (30 cm x 30 cm x 35 cm). A 1 ml tuberculin syringe with a 24-G needle (both by Terumo) was connected to the cannula inserted in the atrium and 300  $\mu$ l of blood was drawn. To prevent clotting, the syringe was filled in with 20  $\mu$ l of saline containing 200 U/ml of heparin beforehand. A stainless-steel microinjection cannula (0.17 mm in. dia., 0.35 mm out. dia., EICOM) connected to Teflon tube (50 cm long, 0.1 mm in. dia., 0.4 mm out. dia., EICOM) was inserted into the guide cannula positioned in the third ventricle. The length of the microinjection cannula was adjusted beforehand so that its tip would be extend 1 mm from the guide cannula. One end of the Teflon tube was connected to a microsyringe pump and either PBS or 19P2-L31 dissolved in PBS was injected, in a total volume of 10  $\mu$ l, into the third ventricle at a flow rate of 2.5  $\mu$ l/min. Ten minutes after initiation of administration into the third ventricle, 5  $\mu$ g/kg GHRH-saline was administered via the cannula inserted into the atrium. Immediately before initiation of intraventricular administration and 10, 20, 30, 40, and 60 minutes after administration of GHRH, 300  $\mu$ l portions of blood were drawn from the jugular vein. Each blood sample was centrifuged (5,000 rpm, 10 min.) and the supernatant (plasma) was



recovered. The concentrations of GH in the plasma were determined by radioimmunoassay.

The results were expressed as a mean  $\pm$  S.E.M. To test for significant difference between the group treated with 19P2-L31 dissolved in PBS and the control group treated with PBS alone, Student's t-test was used. According to the two tailed test,  $p < 0.05$  was assumed to be the minimal level of significance. As shown in Fig. 49, administration of  $5 \mu\text{g/kg}$  of GHRH elevated the plasma GH level. However, when 50 nmol of 19P2-L31 was administered into the third ventricle, the GHRH-induced elevation of plasma GH was significantly inhibited.

[Example 43]

15 Preparation of rabbit anti-bovine 19P2-L31 antibodies

Synthetic peptides containing partial 19P2-L31 sequence [peptide-I: SRAHQHSMEIRTPDC (SEQ ID NO:92), peptide-II: CAWYAGRGIRPVGRFNH2 (SEQ ID NO:93), and peptide-III: CEIRTPDINPAWYAG (SEQ ID NO:94) were conjugated with KLH according to the standard method. Each peptide conjugate ( $600 \mu\text{g}$  as a peptide) dissolved in saline was mixed with Freund's complete adjuvant, and the resultant emulsion was subcutaneously injected into three rabbits (NZW, male, 2.5 kg) respectively. Hyperimmunization was carried out three times in total at the same dose of the conjugate as the first injection with Freund's incomplete adjuvant every three weeks. Antibody titers were determined as follows. Two weeks after the last immunization, blood samples were obtained from the vein of the immunized rabbits respectively. After being incubated at  $37^\circ\text{C}$  for 1 hour, the blood samples were kept at  $4^\circ\text{C}$  over night. Sera were then prepared by means of centrifugation. An aliquot ( $100 \mu\text{l}$ ) of each serum sample diluted properly was introduced into 96-well polystyrene microplates

which were pre-coated with goat anti-rabbit IgG (Fc) antibodies, and then the microplates were incubated at 4°C for 16 hours. After removing the sera, horse radish peroxidase (HRP)-conjugated peptide-I, II, and III were added to the wells respectively, and then the microplates were incubated at room temperature for 4 hours. After removing the peptides, coloring reaction was done by adding a substrate. The reaction was stopped by adding 100  $\mu$ l of a stopping solution, and then the absorbance at 450 nm in each well was measured. As shown in Fig. 50, serum samples obtained from the rabbits after the immunization showed binding activities to HRP-conjugated peptides respectively. However, none of binding activities was detected in sera prepared before the immunization. These results indicated that the rabbits received the immunization produced antibodies against peptide-I, II, and III, respectively. To prepare purified IgG antibody fractions, sera obtained from the immunized rabbits was precipitated with ammonium sulfate. The resultant precipitates were dissolved in borate buffer, and then dialyzed with the same buffer. The IgG fractions thus obtained were then subjected onto affinity columns conjugated with peptide-I or 19P2-L31 respectively. After washing the columns with borate buffer and following with acetate buffer (100 mM, pH 4.5), antibodies bound to the column were eluted with glycine buffer (200 mM, pH 2.0). After being neutralized with 1M Tris, the eluents were used as purified antibodies respectively.

[Example 44]

Inhibitory activity of antibodies against the release of arachidonic acid metabolites induced by 19P2-L31

The purified antibodies prepared as described in Example 43 were tested their inhibitory activity

against the release of arachidonic acid metabolites induced by 19P2-L31. The antibodies diluted as indicated in Fig. 51 were mixed with 19P2-L31 ( $5 \times 10^{-10}$  M) at room temperature for 1 hour, and then the release of arachidonic acid metabolites was examined as described in Example 11. As shown in Fig. 51, the highest inhibitory activity was observed in anti-peptide-II antibodies.

[Example 45]

Based on the DNA sequence coding the murine-derived ligand polypeptide (Figure 32) obtained in Example 29, two primers, were synthesized.  
rFBG: 5'-AGATTGGCATCATCCAGGAAGACGGAGCAT-3' (SEQ ID NO:95)  
rRSA: 5'-GTCGACTCAGCAGCACTGTCTTCTCGAGCTG-3' (SEQ ID NO:96)

Using the cDNA prepared using 0.5 ng of m01.2212.....121urine genomic DNA (Mouse BALB/c genomic DNA as a template and PCR was

carried out.

50  $\mu$ l of reaction mixture comprises 200nM each of synthetic DNA primer, 0.5 nM of template DNA, 0.25mM of dNTPs, 0.5  $\mu$ l of E X Taq polymerase, and buffers attached with enzyme. An amplification reaction was carried out in 30 cycles of 95°C x 30 sec and 67°C x 60 sec. The amplification product was identified by 1.2% agarose gel electrophoresis with ethidium bromide staining and a 1 kb (approx.) band was recovered and subcloned using TA Cloning Kit (Invitrogen). This ligation mixture was used to transform E. coli JM109 and clones harboring the inserted fragment were selected on ampicillin- and X-gal-containing LB agar. A white clone was isolated to provide a transformant, Escherichia coli JM109/pmGB3. This clone was cultured overnight in an ampicillin-containing LB medium and, using an automatic plasmid extractor, a plasmid DNA was

prepared. A portion of the DNA thus prepared was subjected to a sequencing reaction using ABI Dye Terminator Cycle Sequencing Kit (ABI) and analyzed with a fluorescent automated sequencer. The oligonucleotide sequence data thus obtained was analyzed with DNASIS (Hitachi System Engineering) (Fig. 53). The underscored sequences correspond to the primer sequences.

The nucleotide sequence determined in this manner was compared with the sequence of SEQ ID NO:2, 46, or 60. As a result, the DNA fragment inserted in the plasmid pmGB3 harbored by *Escherichia coli* JM109/pmGB3 was found to code for a novel mouse ligand polypeptide [Fig. 54].

[Example 46]

The influence of 19P2-L31 on prolactin secretion from pituitary cell line RC-4B/C

The rat pituitary cell line RC-4B/C (Hurbain-Kosmath et al., In Vitro Cell. Dev. Biol., 26, 431-440 (1990)) was seeded on a 12-well plate (Sumitomo Bakelite) at a density of  $1 \times 10^5$  cells/well and cultured for 2 days. The medium composition was as suggested in the above literature (DMEM (Nissui):  $\alpha$ -MEM (Gibco) = 1:1, 10% fetal calf serum, 1.5 g/l glucose (Wako), 0.2 mg/ml BSA (Sigma), 0.5% nonessential amino acids solution (Flow Laboratories), 15 mM HEPES (Wako) pH 7.3, 2.5 ng/ml EGF (Genzyme), 50 ng/ml gentamicin (Gibco)) and the cultivation was carried out under 10% CO<sub>2</sub> at 34°C.

The cultured cells were washed with 3 portions of incubation buffer (DMEM:  $\alpha$ -MEM = 1:1, 0.5 g/l glucose, 0.1% BSA, 0.5% nonessential amino acids solution, 15 mM HEPES pH 7.3) and after addition of the same buffer, a preincubation was carried out under 10% CO<sub>2</sub> at 34°C for 15 minutes. The cells were re-washed with two portions of the same buffer. Then, a preparation of bovine

19P2-L31 peptide (SEQ ID NO:5) in incubation buffer was added at the varying concentration shown in Fig. 55 and an incubation was performed under 10% CO<sub>2</sub> at 34°C for 30 minutes. To remove the floating cells, the culture was centrifuged with a high-speed microcentrifuge and the supernatant was stored at -30°C.

The amount of prolactin in the culture supernatant sample obtained by the above procedure was determined with Rat Prolactin [<sup>125</sup>I] Assay System (Amersham).

It can be seen from Fig. 55 that addition of 19P2-L31 caused a concentration-dependent increase in prolactin secretion from RC-4B/C cells. The mark \*\* in the diagram indicates a significance with not less than 99% confidence versus the experiment without addition of 19P2-L31 as analyzed by Student's t-test.

[Example 47]

The influence of 19P2-L31 on prolactin secretion from primary cultured rat pituitary cells

The primary cultured rat pituitary cells were prepared according to the method of Shiota et al. (Acta Endocrinologica, 106, 71-78 (1984)).

A female Fischer 344/N rat (SLC) at about 11 lactation days was decapitated to death and the anterior lobe of hypophysis was isolated. The isolated pituitary specimen was washed with buffer A [137 mM NaCl (Wako), 5 mM KCl (Wako), 0.7 mM Na<sub>2</sub>HPO<sub>4</sub> (Wako), 50 μg/ml gentamicin (Gibco)] and treated with enzyme solution I [0.4% collagenase A (Boehringer-Mannheim), 10 μg/ml DNase (Sigma), 0.4% BSA (Sigma), 0.2% glucose (Wako)] in buffer A at 37°C for 1 hour. After the pituitary preparation was dispersed into cells by pipetting, the dispersion was centrifuged to remove the supernatant and the pellet was suspended in enzyme solution II (0.25% pancreatin (Sigma) in buffer A and incubated at 37°C for 8 minutes. The reaction was

stopped by adding fetal calf serum and the reaction mixture was centrifuged to remove the supernatant. The resulting cells were suspended in DMEM-I (DMEM: Dulbecco's minimum essential medium, 10% fetal calf serum, 20 mM HEPES pH 7.3, 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin), passed through a cell strainer (Falcon) to remove cell conglomerates and fibrous contaminants, and washed with 2 portions of DMEM-I. The cells thus obtained were diluted in DMEM-I, seeded at a cell density of  $1.5 \times 10^5$ /well, and cultured under 5% CO<sub>2</sub> at 37°C for 4 days.

On day 3 of culture the medium was replaced with fresh one and on day 4 a sample of culture supernatant was prepared. Thus, cells were washed with 3 portions of DMEM-II (DMEM, 0.2% BSA, 20 mM HEPES pH 7.3), DMEM-II was added, and the mixture was preincubated under 5% CO<sub>2</sub> at 37°C for 1 hour. After washing with 2 portions of DMEM-II, a solution of 19P2-L31 peptide (amide form of SEQ ID NO:5) in DMEM-II was added at the varying concentration shown in Fig. 56 and the reaction was carried out under 5% CO<sub>2</sub> at 37°C for 1 hour. The supernatant was recovered, centrifuged to remove floating cells, and stored at -30°C for use as a supernatant sample.

The concentration of prolactin in the culture supernatant was determined with Rat Prolactin [<sup>125</sup>I] Assay System (Amersham).

It can be seen from Fig. 56 that addition of 19P2-L31 caused a concentration-dependent increase in prolactin secretion from the primary cultured pituitary cells. The mark \*\* in the diagram indicates that as analyzed by Student's t-test the particular value is statistically significant at  $p < 0.01$  compared with the corresponding value found without addition of 19P2-L31.

The mark \* indicates that as analyzed by Student's t-test the particular value is significant at  $p < 0.05$

compared with the corresponding value found without addition of 19P2-L31.

[Example 48]

The time course of expression of UHR-1 gene in the rat placenta

From female rats at 12 weeks of age, placental samples were isolated on days 11, 14, 17, and 20 of gestation. Those tissues were quickly frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . For the preparation of mRNA, each frozen tissue was homogenized with Isogen solution (Nippon Gene) and then total RNA was prepared in accordance with its manual. From 1 mg of each total RNA, mRNA was prepared using a mRNA Purification Kit (Pharmacia).

After 1  $\mu\text{g}$  of the mRNA was treated with DNase I (Amprification Grade, Gibco BRL), 160 ng was taken and synthesized a cDNA using a RNA PCR Kit (Takara Shuzo) with random 9mer primers at  $42^{\circ}\text{C}$  for 30 minutes. Each of the cDNAs thus prepared was dissolved in 40  $\mu\text{l}$  of TE buffer. Assay of the amount of expression of UHR-1 gene was carried out using ABI PRISM 7700 Sequence Detector (Perkin-Elmer). For the reaction, rU1F (5'-AACCCCTTCATCTATGCGTGG-3') and rU1R (5'-

ATATTCTGGCCATGAGGCAC-3' (SEQ ID NO:98)) were used as primers and rU1P (5'-TTCCGAGAGGAGCTACGCAAGATGCTTC-3' (SEQ ID NO:99)) as the fluorescence-labeled probe.

The reaction mixture was prepared using the proprietary reagent kit TaqMan PCR Core Reagent Kit (Perkin-Elmer) in accordance with the manual. In this procedures, 4  $\mu\text{l}$  of a 40-fold dilution of the sample cDNA in TE buffer was added to the reaction mixture. A DNA fragment for which the number of moles of UHR-1 gene was determined by measuring the absorbance at 260 nm was diluted, and then used as templates for PCR to obtain a calibration curve for quantification. PCR was performed under the conditions of  $50^{\circ}\text{C}$  x 2 min. and

95°C x 10 min, followed by 40 cycles of 95°C x 10 sec.  
and 55°C x 1.5 min. The results indicated that the  
amount of expression of UHR-1 gene in the rat placenta  
increased remarkably with an increasing gestation  
period.

[Example 49]

The influence of 19P2-L31 on plasma prolactin  
concentration in rats

(1) Activity of 19P2-L31 on male rats

The inventors studied the influence of 19P2-L31  
administered i.v. on plasma prolactin concentration on  
male rats. Mature male Fischer rats (body weights: ca  
150-180 g) were anesthetized with urethane 1.5 mg/kg  
i.p. and each sides of the right jugular vein were  
exposed by operation, 20 minutes after anesthesia. 15  
minutes after the operation, a solution of 19P2-L31 (50  
or 500 nmol/kg) in 1% bovine serum albumin (BSA)-saline  
or, in the control group, 1% BSA-saline was  
administered by using a 1 ml tuberculin syringe.  
Immediately before initiation of intravenous  
administration and 2, 5, 10, and 20 minutes after  
administration, 200  $\mu$ l of blood was serially drawn  
from the jugular vein. To prevent clotting, the  
syringe was filled in with 10  $\mu$ l of 150 U/ml heparin-  
saline ahead of time. Each blood sample was  
centrifuged (10,000 rpm, 15 min.) using a high-speed  
refrigerated microcentrifuge (MR-150, Tomy Precision  
Machinery) and the supernatant (plasma) was recovered.  
The amount of prolactin contained in the plasma was  
determined with a radioimmunoassay kit (Amersham). The  
time course of plasma prolactin concentration was  
expressed in mean  $\pm$  S.E.M. and the significance of  
difference between the 19P2-L31/1% BSA-saline group and  
the 1% BSA-saline group was tested by Dunnett's method.  
The 5% level of significance ( $p \leq 0.05$ ) was used. It is  
clear from Fig. 58 that administration of 19P2-L31 in a



dose of 500 nmol/kg caused a significant increase in plasma prolactin concentration, compared with the control group, at 2 minutes following administration.

(2) Activity of 19P2-L31 on female rats

5           Subsequently, the inventors studied the influence of 19P2-L31 administered i.v. on plasma concentration on female rats. Sexual cycles of mature female Fischer rats (body weight : ca 140 to 160g) were determined by ostium vaginae test, and the influence of 19P2-L31  
10 administered i.v. on plasma prolactin concentration was studied by the same method as described on Example 49-(1) mentioned above. The time course of plasma prolactin concentration was expressed in mean  $\pm$  S.E.M. and the significance of difference between the 19P2-L31/1% BSA-saline group and the 1% BSA-saline group was  
15 tested by Dunnett's method. The 5% level of significance ( $p \leq 0.05$ ) was used. It is clear from Fig. 59 that administration of 19P2-L31 in a dose of 50 nmol/kg caused a significant increase in plasma prolactin concentration, compared with the control  
20 group, at 5 minutes following administration. It is also clear from Fig. 59 that administration of 19P2-L31 on female rats in a dose of about 1/10 showed the equivalent or superior activities compared with the case of the administration on male rats. In addition,  
25 As shown in Fig. 60, when the time course of plasma prolactin concentration was determined among the sexual cycle, a significant increase in plasma prolactin concentration was observed in estrus. This indicates that the effect of 19P2-L31 is different depending on  
30 the sexual cycles of the female rats.

[Preparation Example 1]

Fifty milligrams of the compound as obtained in Example 21 is dissolved in 50 ml of distilled water for  
35 injection (Japanese pharmacopoeial), and distilled water for injection (Japanese pharmacopoeial) is added

thereto to make 100 ml. The resulting solution is filtered under a germ-free condition, and each vial for injection is filled in with 1 ml of the filtrate, freeze-dried and sealed therein also under a germ-free condition.

[Preparation Example 2]

One hundred milligrams of the compound as obtained in Example 21 is dissolved in 50 ml of distilled water for injection (Japanese pharmacopoeial), and distilled water for injection (Japanese pharmacopoeial) is added thereto to make 100 ml. The resulting solution is filtered under a germ-free condition, and each vial for injection is filled in with 1 ml of the filtrate, freeze-dried and sealed therein also under a germ-free condition.

[Sequence Listing]

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Takeda Chemical Industries, Ltd.
- (B) STREET: 1-1, Doshomachi 4-chome, Chuo-ku
- (C) CITY: Osaka
- (D) STATE: Osaka
- (E) COUNTRY: Japan
- (F) POSTAL CODE (ZIP): 541

(ii) TITLE OF INVENTION: Polypeptides, Their Production and Use

(iii) NUMBER OF SEQUENCES: 94

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE:
- (B) COMPUTER:
- (C) OPERATING SYSTEM:
- (D) SOFTWARE:

(v) CURRENT APPLICATION DATA:

APPLICATION NUMBER:

(A) LENGTH: 98

(C) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

10            1                            5                            10                            15

**20                      25                      30**

**35                      40                      45**

**50                      55                      60**

**65                      70                      75                      80**

20 85 90 95

(2) INFORMATION FOR SEQ ID NO:2:

(A) LENGTH: 294

(C) STRANDENESS: Double

(11) MOLECULE TYPE: cDNA

(C) IDENTIFICATION METHOD: S

(x) SEQUENCE DESCRIPTION; SEQ ID NO:2:

35 GCTGCCAGCA GAGCCCACCA GCACTCCATG GAGATCCGCA CCCCCGACAT CAACCCTGCC 120

TTGTTACGCRG GCGGTGGGAT COGGCCCGTG GGCGGCTTCG GCGGGCGAAG AGGTGCCCYG 180

GGGGACGGAC CCAGGCCTGG CCCCCGGCGT GTGCCGGCCT GCTTCGCGCT GGAAGGOGGY 240  
GCTGAGCCCT CCGAGCCCT CCGGGGGGG CTGACGGCCC AGCTGGTCCA GGAA 294

(2) INFORMATION FOR SEQ ID NO:3:

- 5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29  
(B) TYPE: Amino acid  
(C) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Peptide  
10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ser Arg Ala His Gln His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn  
1 5 10 15  
Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro Val Gly  
15 20 25

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 19  
20 (B) TYPE: Amino acid  
(C) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Peptide  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

25 Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro  
1 5 10 15  
Val Gly Arg  
19

30 (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31  
(B) TYPE: Amino acid  
(C) TOPOLOGY: Linear  
35 (ii) MOLECULE TYPE: Peptide  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

135

Ser Arg Ala His Gln His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn  
1 5 10 15  
Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro Val Gly Arg Phe  
5 20 25 30

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32  
10 (B) TYPE: Amino acid  
(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

15 Ser Arg Ala His Gln His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn  
1 5 10 15  
Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro Val Gly Arg Phe Gly  
20 25 30

## 20 (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33  
(B) TYPE: Amino acid  
(C) TOPOLOGY: Linear

25 (ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ser Arg Ala His Gln His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn  
1 5 10 15  
30 Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro Val Gly Arg Phe Gly  
20 25 30

Arg

33

## 35 (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

136

(A) LENGTH: 20

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro

1 5 10 15

Val Gly Arg Phe

10 20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

15 (B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

20 Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro

1 5 10 15

Val Gly Arg Phe Gly

20

25 (2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

30 (ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro

1 5 10 15

35 Val Gly Arg Phe Gly Arg

20

137

## (2) INFORMATION FOR SEQ ID NO:11:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 87

5 (B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

10

AGCAGAGCCC ACCAGCACTC CATGGAGATC CGCACCCCGG ACATCAACCC TGCCTGGTAC 60  
GCRGGCCGTG GGATCCGGCC CGTGGGC 87

## (2) INFORMATION FOR SEQ ID NO:12:

## 15 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 57

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

20 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ACCCCGACA TCAACCTGC CTGGTAOGR GGCCGTGGGA TCCGGCCGT GGGCCGC 57

## 25 (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 93

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

30 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AGCAGAGCCC ACCAGCACTC CATGGAGATC CGCACCCCGG ACATCAACCC TGCCTGGTAC 60  
35 GCRGGCCGTG GGATCCGGCC CGTGGGCCG TTC 93

138

## (2) INFORMATION FOR SEQ ID NO:14:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 96  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

10 AGCAGAGCCC ACCAGCACTC CATGGAGATC CGCACCCCGG ACATCAACCC TGCCTGGTAC 60  
GCRGGCCGTG GGATCOGGCC CGTGGGCOGC TTCGGC 96

## (2) INFORMATION FOR SEQ ID NO:15:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 99  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

15 AGCAGAGCCC ACCAGCACTC CATGGAGATC CGCACCCCGG ACATCAACCC TGCCTGGTAC 60  
GCRGGCCGTG GGATCOGGCC CGTGGGCOGC TTCGGCCGG 99

## 25 (2) INFORMATION FOR SEQ ID NO:16:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 60  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

30 ACCCCGACA TCAACCCTGC CTGGTAOCGR GGCCGTGGGA TCOGGCCCGT GGGCCGCTTC 60

## (2) INFORMATION FOR SEQ ID NO:17:



139

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 63  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ACCCCGACA TCAACCTGC CTGGTACGCR GCGGTGGGA TCGGCCCGT GGGCGCTTC 60

10 GGC

63

## (2) INFORMATION FOR SEQ ID NO:18:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 66  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

ACCCCGACA TCAACCTGC CTGGTACGCR GCGGTGGGA TCGGCCCGT GGGCGCTTC 60

GGCOGG

66

## (2) INFORMATION FOR SEQ ID NO:19:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 91  
(B) TYPE: Amino acid  
(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Leu Val Leu Val Ile Ala Arg Val Arg Arg Leu His Asn Val Thr Asn

1

5

10

15

Phe Leu Ile Gly Asn Leu Ala Leu Ser Asp Val Leu Met Cys Thr Ala

35

20

25

30

Cys Val Pro Leu Thr Leu Ala Tyr Ala Phe Glu Pro Arg Gly Trp Val

140

35                      40                      45  
 Phe Gly Gly Gly Leu Cys His Leu Val Phe Phe Leu Gln Pro Val Thr  
 50                      55                      60  
 Val Tyr Val Ser Val Phe Thr Leu Thr Thr Ile Ala Val Asp Arg Tyr  
 5      65                      70                      75                      80  
 Val Val Leu Val His Pro Leu Arg Arg Arg Ile  
                     85                      90

- (2) INFORMATION FOR SEQ ID NO:20:
- 10      (1) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 59
- (B) TYPE: Amino acid
- (C) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: Peptide
- 15      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Gly Leu Leu Leu Val Thr Tyr Leu Leu Pro Leu Leu Val Ile Leu Leu  
 1                      5                      10                      15  
 Ser Tyr Val Arg Val Ser Val Lys Leu Arg Asn Arg Val Val Pro Gly  
 20                      20                      25                      30  
 Cys Val Thr Gln Ser Gln Ala Asp Trp Asp Arg Ala Arg Arg Arg Arg  
                     35                      40                      45  
 Thr Phe Cys Leu Leu Val Val Val Val Val Val  
 50                      55

- 25
- (2) INFORMATION FOR SEQ ID NO:21:
- (1) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 370
- (B) TYPE: Amino acid
- 30          (C) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: Peptide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Met Ala Ser Ser Thr Thr Arg Gly Pro Arg Val Ser Asp Leu Phe Ser  
 35      1                      5                      10                      15  
 Gly Leu Pro Pro Ala Val Thr Thr Pro Ala Asn Gln Ser Ala Glu Ala

141

	20	25	30
	Ser Ala Gly Asn Gly Ser Val Ala Gly Ala Asp Ala Pro Ala Val Thr		
	35	40	45
	Pro Phe Gln Ser Leu Gln Leu Val His Gln Leu Lys Gly Leu Ile Val		
5	50	55	60
	Leu Leu Tyr Ser Val Val Val Val Val Gly Leu Val Gly Asn Cys Leu		
	65	70	75
	Leu Val Leu Val Ile Ala Arg Val Arg Arg Leu His Asn Val Thr Asn		
	85	90	95
10	Phe Leu Ile Gly Asn Leu Ala Leu Ser Asp Val Leu Met Cys Thr Ala		
	100	105	110
	Cys Val Pro Leu Thr Leu Ala Tyr Ala Phe Glu Pro Arg Gly Trp Val		
	115	120	125
	Phe Gly Gly Gly Leu Cys His Leu Val Phe Phe Leu Gln Pro Val Thr		
15	130	135	140
	Val Tyr Val Ser Val Phe Thr Leu Thr Thr Ile Ala Val Asp Arg Tyr		
	145	150	155
	Val Val Leu Val His Pro Leu Arg Arg Arg Ile Ser Leu Arg Leu Ser		
	165	170	175
20	Ala Tyr Ala Val Leu Ala Ile Trp Ala Leu Ser Ala Val Leu Ala Leu		
	180	185	190
	Pro Ala Ala Val His Thr Tyr His Val Glu Leu Lys Pro His Asp Val		
	195	200	205
	Arg Leu Cys Glu Glu Phe Trp Gly Ser Gln Glu Arg Gln Arg Gln Leu		
25	210	215	220
	Tyr Ala Trp Gly Leu Leu Leu Val Thr Tyr Leu Leu Pro Leu Leu Val		
	225	230	235
	Ile Leu Leu Ser Tyr Val Arg Val Ser Val Lys Leu Arg Asn Arg Val		
	245	250	255
30	Val Pro Gly Cys Val Thr Gln Ser Gln Ala Asp Trp Asp Arg Ala Arg		
	260	265	270
	Arg Arg Arg Thr Phe Cys Leu Leu Val Val Val Val Val Phe Ala		
	275	280	285
	Val Cys Trp Leu Pro Leu His Val Phe Asn Leu Leu Arg Asp Leu Asp		
35	290	295	300
	Pro His Ala Ile Asp Pro Tyr Ala Phe Gly Leu Val Gln Leu Leu Cys		

142

305                      310                      315                      320  
 His Trp Leu Ala Met Ser Ser Ala Cys Tyr Asn Pro Phe Ile Tyr Ala  
                          325                      330                      335  
 Trp Leu His Asp Ser Phe Arg Glu Glu Leu Arg Lys Leu Leu Val Ala  
 5                      340                      345                      350  
 Trp Pro Arg Lys Ile Ala Pro His Gly Gln Asn Met Thr Val Ser Val  
                          355                      360                      365  
 Val Ile  
                          370

10

## (2) INFORMATION FOR SEQ ID NO:22:

## (1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 206

(B) TYPE: Amino acid

15

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Leu Val Leu Val Ile Ala Arg Val Arg Arg Leu Tyr Asn Val Thr Asn  
 20                      1                      5                      10                      15  
 Phe Leu Ile Gly Asn Leu Ala Leu Ser Asp Val Leu Met Cys Thr Ala  
                          20                      25                      30  
 Cys Val Pro Leu Thr Leu Ala Tyr Ala Phe Glu Pro Arg Gly Trp Val  
                          35                      40                      45  
 25                      Phe Gly Gly Gly Leu Cys His Leu Val Phe Phe Leu Gln Ala Val Thr  
                          50                      55                      60  
 Val Tyr Val Ser Val Phe Thr Leu Thr Thr Ile Ala Val Asp Arg Tyr  
                          65                      70                      75                      80  
 Val Val Leu Val His Pro Leu Arg Arg Arg Ile Ser Leu Arg Leu Ser  
 30                      85                      90                      95  
 Ala Tyr Ala Val Leu Ala Ile Trp Val Leu Ser Ala Val Leu Ala Leu  
                          100                      105                      110  
 Pro Ala Ala Val His Thr Tyr His Val Glu Leu Lys Pro His Asp Val  
                          115                      120                      125  
 35                      Arg Leu Cys Glu Glu Phe Trp Gly Ser Gln Glu Arg Gln Arg Gln Leu  
                          130                      135                      140

143

Tyr Ala Trp Gly Leu Leu Leu Val Thr Tyr Leu Leu Pro Leu Leu Val  
 145                      150                      155                      160  
 Ile Leu Leu Ser Tyr Ala Arg Val Ser Val Lys Leu Arg Asn Arg Val  
                     165                      170                      175  
 5 Val Pro Gly Arg Val Thr Gln Ser Gln Ala Asp Trp Asp Arg Ala Arg  
                     180                      185                      190  
 Arg Arg Arg Thr Phe Cys Leu Leu Val Val Val Val Val Val  
                     195                      200                      205

## 10 (2) INFORMATION FOR SEQ ID NO:23:

## (1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 126

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

## 15 (ii) MOLECULE TYPE: Peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Val Val Leu Val His Pro Leu Arg Arg Arg Ile Ser Leu Arg Leu Ser  
 1                      5                      10                      15  
 20 Ala Tyr Ala Val Leu Gly Ile Trp Ala Leu Ser Ala Val Leu Ala Leu  
                     20                      25                      30  
 Pro Ala Ala Val His Thr Tyr His Val Glu Leu Lys Pro His Asp Val  
                     35                      40                      45  
 Ser Leu Cys Glu Glu Phe Trp Gly Ser Gln Glu Arg Gln Arg Gln Ile  
 25                      50                      55                      60  
 Tyr Ala Trp Gly Leu Leu Leu Gly Thr Tyr Leu Leu Pro Leu Leu Ala  
 65                      70                      75                      80  
 Ile Leu Leu Ser Tyr Val Arg Val Ser Val Lys Leu Arg Asn Arg Val  
                     85                      90                      95  
 30 Val Pro Gly Ser Val Thr Gln Ser Gln Ala Asp Trp Asp Arg Ala Arg  
                     100                      105                      110  
 Arg Arg Arg Thr Phe Cys Leu Leu Val Val Val Val Val Val  
                     115                      120                      125

## 35 (2) INFORMATION FOR SEQ ID NO:24:

## (1) SEQUENCE CHARACTERISTICS:

144

- (A) LENGTH: 273  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear  
5 (ii) MOLECULE TYPE: cDNA  
(ix) FEATURE  
(C) IDENTIFICATION METHOD: S  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

10 CTGGTGCTGG TGATOGGCG GGTGGGCGG CTGCACAACG TGACGAACTT CCTCATCGGC 60  
AACCTGGCCT TGTCGGACGT GCTCATGTGC ACGGCGTCGG TGGCGCTCAC GCTGGCCTAT 120  
GCCTTCGAGC CACGGGGCTG GGTGTTGGG GGGGGCTGT GCCACCTGGT CTTCTTCCTG 180  
CAGCCGGTCA CGTCTATGT GTCGGTGTTC ACGCTACCA CCATCGCAGT GGACCGGTAC 240  
GTCGTGCTGG TGCACCCGCT GAGGCGGCGC ATC 273

15

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 177  
(B) TYPE: Nucleic acid  
20 (C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

30

GGCCTGCTGC TGGTCACCTA CCTGCTCCCT CTGCTGGTCA TCCTCCTGTC TTACGTCCGG 60  
GTGTCAGTGA AGCTCCGCAA CCGGTGGTG CCGGGCTGCG TGACCCAGAG CCAGGCGGAC 120  
TGGGACCGCG CTGGGCGCG GCGCACCTTC TGCTTGCTGG TGGTGGTGGT GGTGGTG 177

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1110  
(B) TYPE: Nucleic acid  
35 (C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

145

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

5

ATGGCCTCAT CGACCACTCG GGGCCCCAGG GTTTCGACT TATTTTCCTGG GCTGCGCCG 60  
GCGGTCACAA CTCCCGCCAA CCAGAGCGCA GAGGCCTGG CGGGCAACGG GTGGGTGGCT 120  
GGGCGGACG CTCAGCGGT CAOGCCCTTC CAGAGCCTGC AGCTGGTGCA TCAGCTGAAG 180  
GGGCTGATCG TGCTGCTCTA CAGCGTGTG GTGGTGTGG GGCTGGTGG CAACTGCCTG 240  
CTGGTGCTGG TGATCGGCG GGTGCGCCG CTGCACAACG TGACGAACCT CCTCATCGGC 300  
AACCTGGCCT TGTCGACGT GCTCATGTGC ACCGCCTGG TGCGGCTCAC GCTGGCCTAT 360  
GCCTTGAGC CAGCGGGCTG GGTGTTGGC GGGGCTGT GCCACCTGGT CTTCTTCTG 420  
CAGCGGTCA CGTCTATGT GTGGTGTTC ACGCTACCA CCATGCGAGT GGACGCTAC 480  
GTGTGCTGG TGACCGCGT GAGCGGGCG ATCTCGCTGC GCCTCAGCG CTACGCTGTG 540  
CTGGCCATCT GGGCGCTGTC CGGGTGTG GCGCTGCCG CGCGCGTGCA CACCTATCAC 600  
GTGGAGCTCA AGCGCAACA CGTGCGCTC TCGAGGAGT TCTGGGGCTC CCAGGAGCGC 660  
CAGCGCCAGC TCTAGCCCTG GGGGCTGTG CTGGTCACT ACCTGCTCCC TCTGCTGGT 720  
ATCTCTCTGT CTTAGTCCG GGTGTGAGT AAGCTCGCA ACGCGTGGT GCGGGCTGC 780  
GTGACCCAGA GCCAGGCCA CTGGGACGC GCTGGGCGC GGCGACCTT CTGCTGTCTG 840  
GTGGTGGTGG TGGTGGTGT CGCGCTGTC TGCTGCGC TGCACTCTT CAACCTGCTG 900  
CGGGACCTCG ACCCCACGC CATCGACCT TAOGCCTTG GGCTGGTGCA GCTGCTCTG 960  
CACTGGCTCG CCATGAGTTC GGCTGCTAC AACCCCTCA TCTAGCCTG GCTGCAAGAC 1020  
AGCTTCGCG AGGAGCTCG CAACTGTG GTGCTTGGC CCGCAAGAT AGCCCCCAT 1080  
GGCCAGAATA TGACGTCAG CGTGGTCATC 1110

25

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 618

(B) TYPE: Nucleic acid

30

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

146

CTGGTGCTGG TGATOGGCGG GGTGGGCGG CTGTACAACG TGACGAATTT CCTCATGGC 60  
 AACCTGGCCT TGTCGACGT GCTCATGTGC ACGGCTGGG TGCGGCTCAC GCTGGCCTAT 120  
 GCCTTGGAGC CAGCGGGCTG GGTGTGGGG GGGGGCTGT GCCACCTGGT CTTCTTCCTG 180  
 CAGGCGGTCA CGTCTATGT GTGGGTGTTT ACGCTACCA CCATGCGAGT GGACCGCTAC 240  
 5 GTGGTGCTGG TGCACCGGCT GAGGGGGGCG ATCTGGCTGC GCCTCAGGCG CTACGGCTGTG 300  
 CTGGCCATCT GGGTGCTGTC CGGGTGCTG GCGCTGCCG CGCGGTGCA CACCTATCAC 360  
 GTGGAGCTCA AGCGCACGA CGTGGGCTC TCGAGGAGT TCTGGGGCTC CCAGGAGGCG 420  
 CAGGCGGAGC TCTACGCGTG GGGGCTGCTG CTGGTCACCT ACCTGCTCCC TCTGCTGGTC 480  
 ATCTCTCTGT CTTACGCGCG GGTGTGAGTG AAGCTCGCA ACGGCTGGT GCGGGGCGC 540  
 10 GTGACCCAGA GCCAGGCGA CTGGGACGC GCTGGGGCC GCGCACCTT CTGCTGCTG 600  
 GTGGTGGTGG TGGTGGTG 618

## (2) INFORMATION FOR SEQ ID NO:28:

## (i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 378  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

20 (ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GTGGTCTGG TGCACCGGCT ACGTGGGCG ATTTCACTGA GGCTCAGGCG CTACGGGGTG 60  
 25 CTGGGCATCT GGGCTCTATC TGCACTGCTG GCGCTGCCG CGGGGTGCA CACCTACCAT 120  
 GTGGAGCTCA AGCCCCAGA CGTGAGGCTC TCGAGGAGT TCTGGGGCTC GCAGGAGGCG 180  
 CAAGCCAGA TCTACGCGTG GGGGCTGCTT CTGGGCACCT ATTTGCTCCC CTTGCTGGCC 240  
 ATCTCTCTGT CTTACGTAAG GGTGTGAGTG AAGCTGAGGA ACGGCTGGT GCCTGGCAGC 300  
 GTGACCCAGA GTCAAGCTGA CTGGGACGA GCGGTGGCC GCGCACCTT CTGTCTGCTG 360  
 30 GTGGTGGTGG TGGTAGTG 378

## (2) INFORMATION FOR SEQ ID NO:29:

## (i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 25  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Single



147

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

5

CGTGGSCMTS STGGGCAACN YCTG 25

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

10

(A) LENGTH: 27

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

15

Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GTNGWRRGGC ANCCAGCAGA KGGCAAA 27

20

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

25

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

30

CTGTGYGYSA TYGCNNIKGA YMGSTAC 27

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29

35

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

148

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

5

AKGWAGWAGG GCAGCCAGCA GANSRYGAA 29

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

10

(A) LENGTH: 24

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

15

Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CTGACTTATT TTCTGGGCTG COGC 24

20 (2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

25

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

30 AACACCGACA CATAGACGGT GACC 24

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

35

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

149

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

5

GCICAYCARC AYTGYATGGA 20

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

10

(A) LENGTH: 26

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

15

Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CCIAOGGGIC KDATGCCICK GCCIGC 26

20 (2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

25

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

30 ACGGGCCCKDA TGCCICKGCC IGCRTA 26

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

35

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

150

(D) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

5

COGGCGTACC AGGCAGGGTT 20

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

10

(A) LENGTH: 28  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

AGGCAGGGTT GATGTCGGGG GTGCGGAT 28

20

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

25

(ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

30

CTGCCAGCAG AGOCCACCAG CACTCCA 27

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Single

35

151

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

5

GTGGGGGCCT GGCTCTCTG CCGCTG 27

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

10

(A) LENGTH: 32

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

15

Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

GTGTGACGA ATGAAGGCGG TGGGGCCTG GC 32

20

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

25

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

30

AGGCTCCGCGC TGTATTCCT GGAC 24

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 98

35

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

152

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Met Lys Ala Val Gly Ala Trp Leu Leu Cys Leu Leu Leu Gly Leu  
 5        1                    5                    10                    15  
 Ala Leu Gln Gly Ala Ala Ser Arg Ala His Gln His Ser Met Glu Ile  
                   20                    25                    30  
 Arg Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg  
                   35                    40                    45  
 10    Pro Val Gly Arg Phe Gly Arg Arg Arg Ala Ala Leu Gly Asp Gly Pro  
                   50                    55                    60  
 Arg Pro Gly Pro Arg Arg Val Pro Ala Cys Phe Arg Leu Glu Gly Gly  
                   65                    70                    75                    80  
 15    Ala Glu Pro Ser Arg Ala Leu Pro Gly Arg Leu Thr Ala Gln Leu Val  
                   85                    90                    95  
 Gln Glu

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

20        (A) LENGTH:    83  
           (B) TYPE:     Amino acid  
           (C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

25        Met Ala Leu Lys Thr Trp Leu Leu Cys Leu Leu Leu Ser Leu Val  
           1                    5                    10                    15  
 Leu Pro Gly Ala Ser Ser Arg Ala His Gln His Ser Met Glu Thr Arg  
                   20                    25                    30  
 30    Thr Pro Asp Ile Asn Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro  
                   35                    40                    45  
 Val Gly Arg Phe Gly Arg Arg Arg Ala Thr Pro Arg Asp Val Thr Gly  
                   50                    55                    60  
 Leu Gly Gln Leu Ser Cys Leu Pro Leu Asp Gly Arg Thr Lys Phe Ser  
 35        65                    70                    75                    80  
 Gln Arg Gly

153

## (2) INFORMATION FOR SEQ ID NO:46:

## (1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 249  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

ATGGCCCTGA AGACGTGGCT TCTGTGCTTG CTGCTGCTAA GCTTGGTCCT CCCAGGGGCT 60  
TCCAGCOGAG CCCACCAGCA CTCCATGGAG ACAAGAACCC CTGATATCAA TCCTGCCTGG 120  
TACACGGGCC GCGGGATCAG GCCTGTGGGC CGCTTCGGCA GGAGAAGGGC AACCCOGAGG 180  
GATGTCACTG GACTTGGCCA ACTCAGCTGC CTCCACTGG ATGGACGCAC CAAGTTCTCT 240  
CAGCGTGA 249

## (2) INFORMATION FOR SEQ ID NO:47:

## (1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31  
(B) TYPE: Amino acid  
(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

Ser Arg Ala His Gln His Ser Met Glu Thr Arg Thr Pro Asp Ile Asn  
1 5 10 15  
Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro Val Gly Arg Phe  
20 25 30

## (2) INFORMATION FOR SEQ ID NO:48:

## (1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32  
(B) TYPE: Amino acid  
(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

Ser Arg Ala His Gln His Ser Met Glu Thr Arg Thr Pro Asp Ile Asn  
5        1                      5                      10                      15  
Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro Val Gly Arg Phe Gly  
                20                      25                      30

(2) INFORMATION FOR SEQ ID NO:49:

10 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

[illegible]

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

30

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro  
1 5 10 15  
Val Gly Arg Phe  
20

35

(2) INFORMATION FOR SEQ ID NO:51:



155

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

5 (ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro

1 5 10 15

10 Val Gly Arg Phe Gly

20

## (2) INFORMATION FOR SEQ ID NO:52:

## (i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 22

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

20

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro

1 5 10 15

Val Gly Arg Phe Gly Arg

20

25

## (2) INFORMATION FOR SEQ ID NO:53:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 93

(B) TYPE: Nucleic acid

30 (C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

156

AGCCGAGCCC ACCAGCACTC CATGGAGACA AGAACCCCTG ATATCAATCC TGCCTGGTAC 60  
ACGGGCCGCG GGATCAGGCC TGTGGGCGC TTC 93

## (2) INFORMATION FOR SEQ ID NO:54:

- 5 (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 96  
    (B) TYPE: Nucleic acid  
    (C) STRANDEDNESS: Double  
    (D) TOPOLOGY: Linear  
10 (ii) MOLECULE TYPE: cDNA  
    (ix) FEATURE  
        (C) IDENTIFICATION METHOD: S  
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

15 AGCCGAGCCC ACCAGCACTC CATGGAGACA AGAACCCCTG ATATCAATCC TGCCTGGTAC 60  
ACGGGCCGCG GGATCAGGCC TGTGGGCGC TCGGC 96

## (2) INFORMATION FOR SEQ ID NO:55:

- 20 (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 99  
    (B) TYPE: Nucleic acid  
    (C) STRANDEDNESS: Double  
    (D) TOPOLOGY: Linear  
    (ii) MOLECULE TYPE: cDNA  
25 (ix) FEATURE  
        (C) IDENTIFICATION METHOD: S  
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

30 AGCCGAGCCC ACCAGCACTC CATGGAGACA AGAACCCCTG ATATCAATCC TGCCTGGTAC 60  
ACGGGCCGCG GGATCAGGCC TGTGGGCGC TCGGCAGG 99

## (2) INFORMATION FOR SEQ ID NO:56:

- 35 (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 60  
    (B) TYPE: Nucleic acid  
    (C) STRANDEDNESS: Double

157

(D) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: cDNA  
(ix) FEATURE  
(C) IDENTIFICATION METHOD: S  
5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

ACCCCTGATA TCAATCCTGC CTGGTACACG GGCCGCGGGA TCAGGCCTGT GGGCCGCTTC 60

(2) INFORMATION FOR SEQ ID NO:57:

10 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 63  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

15 (ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

20 ACCCCTGATA TCAATCCTGC CTGGTACACG GGCCGCGGGA TCAGGCCTGT GGGCCGCTTC 60  
GGC 63

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 66  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

30 (ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

35 ACCCCTGATA TCAATCCTGC CTGGTACACG GGCCGCGGGA TCAGGCCTGT GGGCCGCTTC 60  
GGCAGG 66

158

## (2) INFORMATION FOR SEQ ID NO:59:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 87

(B) TYPE: Amino acid

5 (C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

Met Lys Val Leu Arg Ala Trp Leu Leu Cys Leu Leu Met Leu Gly Leu  
 10 1 5 10 15  
 Ala Leu Arg Gly Ala Ala Ser Arg Thr His Arg His Ser Met Glu Ile  
 20 25 30  
 Arg Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Ser Arg Gly Ile Arg  
 35 40 45  
 15 Pro Val Gly Arg Phe Gly Arg Arg Arg Ala Thr Leu Gly Asp Val Pro  
 50 55 60  
 Lys Pro Gly Leu Arg Pro Arg Leu Thr Cys Phe Pro Leu Glu Gly Gly  
 65 70 75 80  
 Ala Met Ser Ser Gln Asp Gly  
 20 85

## (2) INFORMATION FOR SEQ ID NO:60:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 261

25 (B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

30 (C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

ATGAAGGTGC TGAGGGCCTG GCTCCTGTGC CTGCTGATGC TGGGCCTGGC CCTGCGGGGA 60  
 GCTGCAAGTC GTACCCATCG GCACTCCATG GAGATCGCA CCCCTGACAT CAATCCTGCC 120  
 35 TGGTACGCCA GTCGCGGGAT CAGGCCTGTG GGCCGCTTCG GTCGGAGGAG GGCAACCCCTG 180  
 GGGGACGTCC CCAAGCCTGG CTTGCGACCC CGGCTGACCT GCTTCCCCCT GGAAGGCGGT 240

159

GCTATGTCGT CCCAGGATGG C

261

## (2) INFORMATION FOR SEQ ID NO:61:

## (1) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 31  
(B) TYPE: Amino acid  
(C) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Peptide  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

10

Ser Arg Thr His Arg His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn

1 5 10 15

Pro Ala Trp Tyr Ala Ser Arg Gly Ile Arg Pro Val Gly Arg Phe

20 25 30

15

## (2) INFORMATION FOR SEQ ID NO:62:

## (1) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 32  
(B) TYPE: Amino acid  
(C) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Peptide  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

25

Ser Arg Thr His Arg His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn

1 5 10 15

Pro Ala Trp Tyr Ala Ser Arg Gly Ile Arg Pro Val Gly Arg Phe Gly

20 25 30

## (2) INFORMATION FOR SEQ ID NO:63:

## (1) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 33  
(B) TYPE: Amino acid  
(C) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Peptide  
35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

160

Ser Arg Thr His Arg His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn  
1 5 10 15  
Pro Ala Trp Tyr Ala Ser Arg Gly Ile Arg Pro Val Gly Arg Phe Gly  
20 25 30  
5 Arg

## (2) INFORMATION FOR SEQ ID NO:64:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

15 Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Ser Arg Gly Ile Arg Pro  
1 5 10 15  
Val Gly Arg Phe  
20

## 20 (2) INFORMATION FOR SEQ ID NO:65:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

25 (ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Ser Arg Gly Ile Arg Pro  
1 5 10 15  
30 Val Gly Arg Phe Gly  
20

## (2) INFORMATION FOR SEQ ID NO:66:

## (i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 22

(B) TYPE: Amino acid

161

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

5 Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Ser Arg Gly Ile Arg Pro  
1 5 10 15  
Val Gly Arg Phe Gly Arg  
20

10 (2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 93

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

15 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

20

AGTCGTACCC ATGGGCACTC CATGGAGATC CGCACCCCTG ACATCAATCC TGCCTGGTAC 60  
GCCAGTCGCG GGATCAGGCC TGTGGGCCGC TTC 93

(2) INFORMATION FOR SEQ ID NO:68:

25 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 96

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

30 (ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

35 AGTCGTACCC ATGGGCACTC CATGGAGATC CGCACCCCTG ACATCAATCC TGCCTGGTAC 60  
GCCAGTCGCG GGATCAGGCC TGTGGGCCGC TTGGT 96

162

## (2) INFORMATION FOR SEQ ID NO:69:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 99  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

AGTCGTACCC ATGGGCACTC CATGGAGATC CGCACCCCTG ACATCAATCC TGCCTGGTAC 60  
GCCAGTCGCG GGATCAGGCC TGTGGGCGCG TTCGGTCGG 99

## (2) INFORMATION FOR SEQ ID NO:70:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 60  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

ACCCCTGACA TCAATCCTGC CTGGTACGCC AGTCGCGGGA TCAGGCCTGT GGGCGCTTC 60

## (2) INFORMATION FOR SEQ ID NO:71:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 63  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE



163

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

5 ACCCCTGACA TCAATCCTGC CTGGTACGCC AGTCGCGGGA TCAGGCCTGT GGGCCGCTTC 60  
GGT 63

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 66  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: cDNA  
(ix) FEATURE

15 (C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

20 ACCCCTGACA TCAATCCTGC CTGGTACGCC AGTCGCGGGA TCAGGCCTGT GGGCCGCTTC 60  
GGTCGG 66

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 21  
(B) TYPE: Amino acid  
(C) TOPOLOGY: Linear  
(ii) MOLECULE TYPE: Peptide  
(ix) FEATURE: Xaa of the 10th position is Ala or Thr.  
Xaa of the 11th position is Gly or Ser.  
Xaa of the 21st position is H, Gly or  
30 GlyArg.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Xaa Xaa Arg Gly Ile Arg Pro  
1 5 10 15  
35 Val Gly Arg Phe Xaa  
20

## (2) INFORMATION FOR SEQ ID NO:74:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11

5 (B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(ix) FEATURE: Xaa of the 3rd position is Ala or Thr.

Xaa of the 5th position is Gln or Arg.

10 Xaa of the 10th position is Ile or Thr.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

Ser Arg Xaa His Xaa His Ser Met Glu Xaa Arg

1 5 10

15

## (2) INFORMATION FOR SEQ ID NO:75:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26

(B) TYPE: Nucleic acid

20 (C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

25

CARCAYTCCA TGGAGACAAG AACCCC 26

## (2) INFORMATION FOR SEQ ID NO:76:

## (i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 24

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

35 Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

TACCAGGCAG GATTGATACA GGGG 24

(2) INFORMATION FOR SEQ ID NO:77:

- 5 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 25
  - (B) TYPE: Nucleic acid
  - (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Linear
- 10 (ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

GGCATCATCC AGGAAGACGG AGCAT 25

15

(2) INFORMATION FOR SEQ ID NO:78:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 25
  - (B) TYPE: Nucleic acid
  - 20 (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

25

AGCAGAGGAG AGGGAGGGTA GAGGA 25

(2) INFORMATION FOR SEQ ID NO:79:

- (i) SEQUENCE CHARACTERISTICS:
- 30 (A) LENGTH: 22
  - (B) TYPE: Nucleic acid
  - (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA
- 35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

166

ACGTTGGCTTC TGTGCTTGCT GC 22

## (2) INFORMATION FOR SEQ ID NO:80:

- 5 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 25
  - (B) TYPE: Nucleic acid
  - (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Linear
- 10 (ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

GCCTGATCCC GCGCCCGTG TACCA 25

15

## (2) INFORMATION FOR SEQ ID NO:81:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 26
  - (B) TYPE: Nucleic acid
  - 20 (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

25

TTGCCCTTCT CCGCCGAAG CGGCC 26

## (2) INFORMATION FOR SEQ ID NO:82:

- (i) SEQUENCE CHARACTERISTICS:
- 30 (A) LENGTH: 27
  - (B) TYPE: Nucleic acid
  - (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: Other nucleic acid  
35 Synthetic DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

167

GGGGGGGCT GCAAGTCGTA CCCATCG 27

## (2) INFORMATION FOR SEQ ID NO:83:

5

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

10

- (ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

CGGCACTCCA TGGAGATCG CACCCCT 27

15

## (2) INFORMATION FOR SEQ ID NO:84:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

20

- (ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

25

CAGGCAGGAT TGATGTCAGG GGTGCGG 27

## (2) INFORMATION FOR SEQ ID NO:85:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

30

- (ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA

35

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

168

CATGGAGTGC CGATGGGTAC GACTTGC 27

## (2) INFORMATION FOR SEQ ID NO:86:

- 5 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 27
  - (B) TYPE: Nucleic acid
  - (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Linear
- 10 (ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

GGCCTCCTCG GAGGAGCCAA GGGATGA 27

15

## (2) INFORMATION FOR SEQ ID NO:87:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 27
  - (B) TYPE: Nucleic acid
  - 20 (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

25

GGGAAAGGAG CCCGAAGGAG AGGAGAG 27

## (2) INFORMATION FOR SEQ ID NO:88:

- (i) SEQUENCE CHARACTERISTICS:
- 30 (A) LENGTH: 25
  - (B) TYPE: Nucleic acid
  - (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: Other nucleic acid  
35 Synthetic DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

CCTGCTGGCC ATTCTCCTGT CITAC 25

(2) INFORMATION FOR SEQ ID NO:89:

- 5 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 25
  - (B) TYPE: Nucleic acid
  - (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Linear
- 10 (ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

GGGTCCAGGT CCGCAGAAG GTTGA 25

15

(2) INFORMATION FOR SEQ ID NO:90:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 25
  - (B) TYPE: Nucleic acid
  - 20 (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

25

GAAGACGGAG CATGGCCCTG AAGAC 25

(2) INFORMATION FOR SEQ ID NO:91:

- (i) SEQUENCE CHARACTERISTICS:
- 30 (A) LENGTH: 25
  - (B) TYPE: Nucleic acid
  - (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: Other nucleic acid  
35 Synthetic DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

170

GGCAGCTGAG TTGGCCAAGT CCACT 25

## (2) INFORMATION FOR SEQ ID NO:92:

- 5 (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 15  
    (B) TYPE: Amino acid  
    (C) TOPOLOGY: Linear  
    (ii) MOLECULE TYPE: Peptide  
10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

Ser Arg Ala His Gln His Ser Met Glu Ile Arg Thr Pro Asp Cys  
1 5 10 15

## 15 (2) INFORMATION FOR SEQ ID NO:93:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 15  
    (B) TYPE: Amino acid  
    (C) TOPOLOGY: Linear  
20 (ii) MOLECULE TYPE: Peptide  
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

Cys Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro Val Gly Arg Phe  
1 5 10 15

25

## (2) INFORMATION FOR SEQ ID NO:94:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 15  
    (B) TYPE: Amino acid  
30 (C) TOPOLOGY: Linear  
    (ii) MOLECULE TYPE: Peptide  
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

Cys Glu Ile Arg Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly  
35 1 5 10 15



171

## (2) INFORMATION FOR SEQ ID NO:95:

## (1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

AGATTGGCAT CATCCAGGAA GACGGAGCAT 30

## (2) INFORMATION FOR SEQ ID NO:96:

## (1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

GCTGACTOGA CAGCACTGTC TTCTOGAGCT G 31

## (2) INFORMATION FOR SEQ ID NO:97:

## (1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:

AACCCCTTCA TCTATGCGTG G 21

172

## (2) INFORMATION FOR SEQ ID NO:98:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:

ATATTCTGGC CATGAGGCAC 20

## (2) INFORMATION FOR SEQ ID NO:99:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid  
Synthetic DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:99:

TTCCGAGAGG AGCTACGCAA GATGCTTC 28

## CLAIMS

## WHAT IS CLAIMED IS:

1. An agent for modulating prolactin secretion which  
5 comprises a ligand polypeptide, or a salt thereof, for a G protein-coupled receptor protein.
2. An agent as claimed in claim 1, wherein the ligand polypeptide is a polypeptide comprising an amino acid  
10 sequence represented by SEQ ID NO: 73 or a substantial equivalent thereto, or its amide or ester, or a salt thereof.
3. An agent as claimed in claim 2, wherein the  
15 polypeptide comprising an amino acid sequence represented by SEQ ID NO: 73 is a polypeptide comprising an amino acid sequence represented by SEQ ID NO: 5, 8, 47, 50, 61 or 64.
- 20 4. An agent as claimed in claim 1, which is for promoting prolactin secretion.
5. An agent as claimed in claim 1, which is for  
25 inhibiting prolactin secretion.
6. An agent as claimed in claim 4, which is for  
treating or preventing hypoovarianism, gonocyst  
cacogenesis, menopausal syndrome, euthyroid or  
hypometabolism.
- 30 7. An agent as claimed in claim 5, which is for  
treating or preventing pituitary adenomatosis, brain  
tumor, emmeniopathy, autoimmune disease, prolactinoma,  
infertility, impotence, amenorrhea, galactorrhea,  
35 acromegaly, Chiari-Frommel syndrome, Argonz-del  
Castilo syndrome, Forbes-Albright syndrome, lymphoma,

Sheehan syndrome or dyszoospermia.

8. An agent for modulating placental function, which comprises a ligand polypeptide, or a salt thereof, for  
5 a G protein-coupled receptor protein.
9. An agent as claimed in claim 8, which is for treating or preventing choriocarcinoma, hydatid mole, irruption mole, abortion, unthrifty fetus, abnormal  
10 saccharometabolism, abnormal lipidmetabolism or oxytocia.
10. An agent as claimed in claim 4, which is for promoting lactation of domestic mammal.
- 15 11. An agent as described in claim 4, which is for an aphrodisiac.
12. An agent for diagnosing function of prolactin secretion, which comprises a ligand polypeptide, or a  
20 salt thereof, for a G protein-coupled receptor protein.
13. Use of a ligand polypeptide, or a salt thereof, for a G protein-coupled receptor protein for  
25 manufacture of a medicament for modulating prolactin secretion.
14. A method for modulating prolactin secretion in a mammal, which comprises administering to said mammal a  
30 ligand polypeptide, or a salt thereof, for a G protein-coupled receptor protein.
15. Use of a ligand polypeptide, or a salt thereof, for a G protein-coupled receptor protein for  
35 manufacture of a medicament for modulating placental function.

16. A method for modulating placental function in a mammal, which comprises administering to said mammal a ligand polypeptide, or a salt thereof, for a G protein-coupled receptor protein.
- 5

1/61

[Drawing]

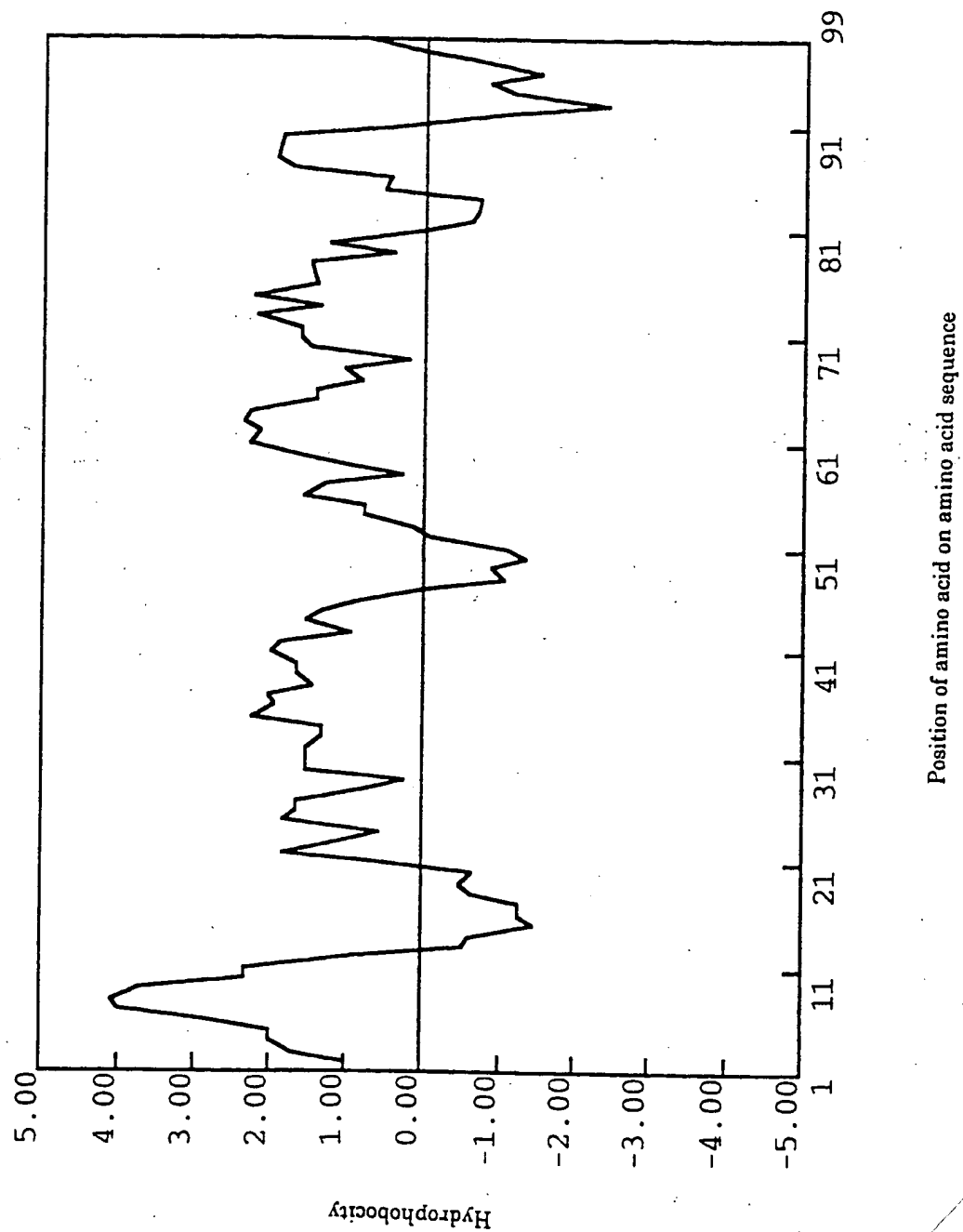
Fig. 1

5'	9	18	27	36	45	54
GTG	GGC	ATG	GTG	GGC	ATC	GGG
---	---	---	---	---	---	---
Val	Gly	Met	Val	Gly	Asn	Val
63	72	81	90	99	108	
CTG	CAC	AAC	GTG	ACG	AAC	CTG
---	---	---	---	---	---	---
Leu	His	Asn	Val	Thr	Asn	Phe
117	126	135	144	153	162	
ATG	TGC	ACC	GCC	TGC	CTC	CTC
---	---	---	---	---	---	---
Met	Cys	Thr	Ala	Cys	Val	Pro
171	180	189	198	207	216	
TGG	GTG	TTC	GGC	GGC	CTG	CTC
---	---	---	---	---	---	---
Trp	Val	Phe	Gly	Gly	Leu	Cys
225	234	243	252	261	270	
GTC	TAT	GTG	TCG	GTG	TTC	ACG
---	---	---	---	---	---	---
Val	Tyr	Val	Ser	Val	Phe	Thr
279	288	297	3'			
CTG	GTG	CAC	COG	CTG	AGG	CGG
---	---	---	---	---	---	---
Leu	Val	His	Pro	Leu	Arg	Arg



3/61

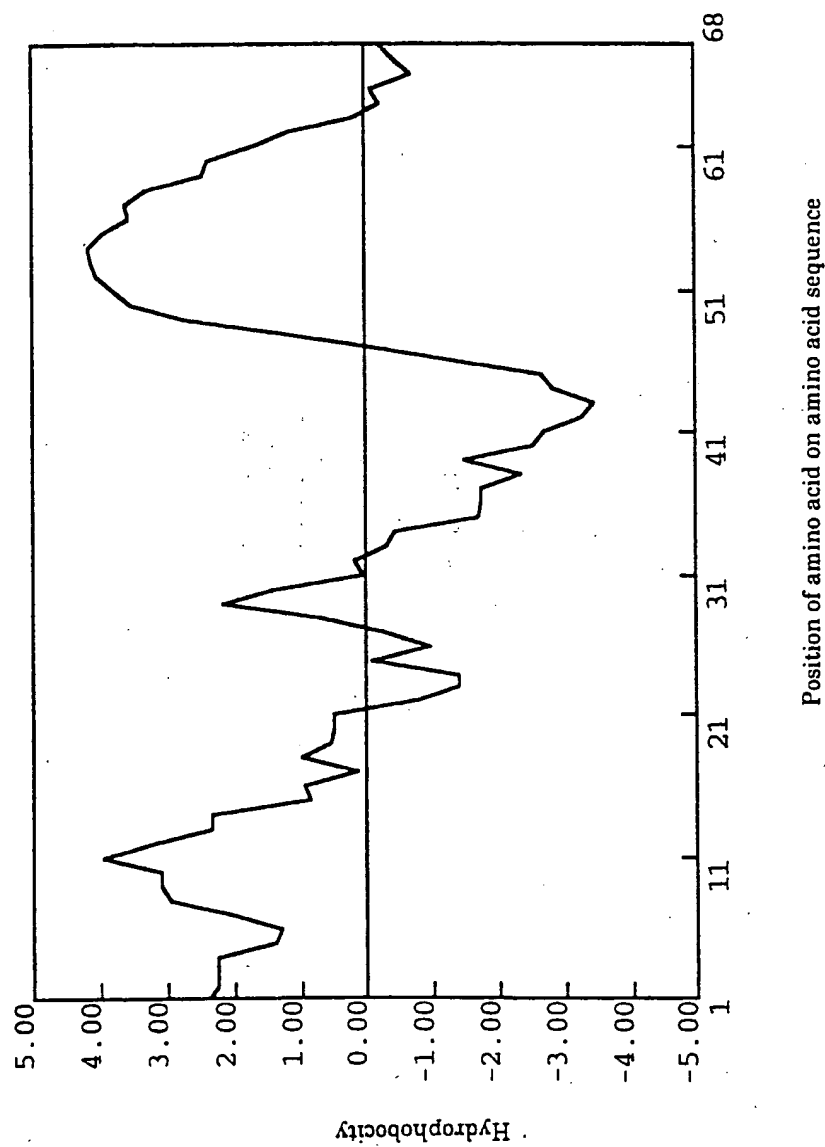
Fig. 3





4/61

Fig. 4



5/61

Fig. 5

p19P2 S12863	10 1	20 1	30 1	40 1	50 1
	VCWVGNVLLV	LVIAVRRLH	NVTNFLICNL	ALSDVLMCTA	CVPLTIAYAF
	LCVSGNLAII	IIILKQKEMR	NVTNLLIVNL	SFSDLAVAM	CLPFTFVYTL
p19P2 S12863	60 51	70 51	80 51	90 51	100 51
	EPRGMVFGCG	LCHLVFELQP	VIVVVSVEFL	TTIAVDRIWV	LVHLELRRRI-
	MDH-MVFEGET	MCKENPEVQC	VSITVSIESL	VLIAVERHQL	IINERGWEPN
p19P2 S12863	110 101	120 101	130 101	140 101	150 101
	NRHAYIGITV	IWVLAVASSL	PFVIYQILTD	EPFQNVSLAA	FKDKYVCFDK
p19P2 S12863	160 151	170 151	180 151	190 151	200 151
	GLLV	TVLPLLVIL	LS-----	VRVSVKLRNR	VWPGCVTQSQ
	FPSDSHRLSY	ITLLVLQYF	GPLCFIFICY	FKIYIRLKR	NNMMDKIRDS
p19P2 S12863	210 201	220 201	230 201	240 201	250 201
	ADWDRARRRR	TFCLLVVVW	VFAICVLEPY	.....	.....
	KYRSSETKRI	NVMLSIWA	-FAVCMLELT	.....	.....

Fig. 6

		9			18			27			36			45			54
5'	GTT	GCC	ATG	GTT	GCC	AAC	ATC	CTG	CTG	CTG	GTT	ATC	GGC	CGG	GTT	CGC	CGG
	Val	Gly	Met	Val	Gly	Asn	Ile	Leu	Leu	Val	Val	Ile	Ala	Arg	Val	Arg	Arg
		63				72			81					99			108
	CTG	TAC	AAC	GTT	ACG	AAT	TTC	CTC	ATC	GGC	AAC	CTG	GCC	TTC	TCC	GAC	GTT
	Leu	Tyr	Asn	Val	Thr	Asn	Phe	Leu	Ile	Gly	Asn	Leu	Ala	Leu	Ser	Asp	Val
			117			126			135			144		153			162
	ATG	TGC	ACC	GCC	TGC	GTT	CCG	CTC	ACG	CTG	GCC	TAT	GCC	TTC	GAG	CCA	CGC
	Met	Cys	Thr	Ala	Cys	Val	Pro	Leu	Thr	Leu	Ala	Tyr	Ala	Phe	Glu	Pro	Gly
			171			180			189			198		207			216
	TGG	GTT	TTC	GGC	GGC	GGC	CTG	TGC	CAC	CTG	GTC	TTC	TTC	CTG	CAG	GCG	GTC
	Trp	Val	Phe	Gly	Gly	Gly	Leu	Cys	His	Leu	Val	Phe	Phe	Leu	Gln	Ala	Val
			225			234			243			252		261			270
	GTC	TAT	GTT	TCG	GTT	TTC	ACG	CTC	ACC	ACC	ATC	GCA	GTT	GAC	CGC	TAC	GTC
	Val	Tyr	Val	Ser	Val	Phe	Thr	Leu	Thr	Thr	Ile	Ala	Val	Asp	Arg	Tyr	Val
			279			288			297			306		315			324
	CTG	GTT	CAC	CCG	CTG	AGG	CGG	CGC	ATC	TGG	CTG	CGC	CTC	AGC	GCC	TAC	GCT
	Leu	Val	His	Pro	Leu	Arg	Arg	Arg	Ile	Ser	Leu	Arg	Leu	Ser	Ala	Tyr	Ala
			333			342			351			360		369			378
	CTG	GCC	ATC	TGG	GTT	CTG	TCC	GCG	GTT	CTG	GCG	CTG	CCC	GCC	GCC	GTT	CAC
	Leu	Ala	Ile	Trp	Val	Leu	Ser	Ala	Val	Leu	Ala	Leu	Pro	Ala	Ala	Val	His
			387			396			405			414		423			432
	TAT	CAC	GTT	GAG	CTC	AAG	CCG	CAC	GAC	GTT	CGC	CTC	TGC	GAG	GAG	TTC	TGG
	Tyr	His	Val	Glu	Leu	Lys	Pro	His	Asp	Val	Arg	Leu	Cys	Glu	Glu	Phe	Trp
			441			450			459			468		477			486
	TCC	CAG	GAG	CGC	CAG	CGC	CAG	CTC	TAC	GCC	TGG	GGG	CTG	CTG	CTG	GTC	ACC
	Ser	Gln	Glu	Arg	Gln	Arg	Gln	Leu	Tyr	Ala	Trp	Gly	Leu	Leu	Leu	Val	Thr
			495			504			513			522		531			540
	CTG	CTC	CCT	CTG	CTG	GTC	ATC	CTC	CTG	TCT	TAC	GCC	CGG	GTT	TCA	GTT	AAG
	Leu	Leu	Pro	Leu	Leu	Val	Ile	Leu	Leu	Ser	Tyr	Ala	Arg	Val	Ser	Val	Lys

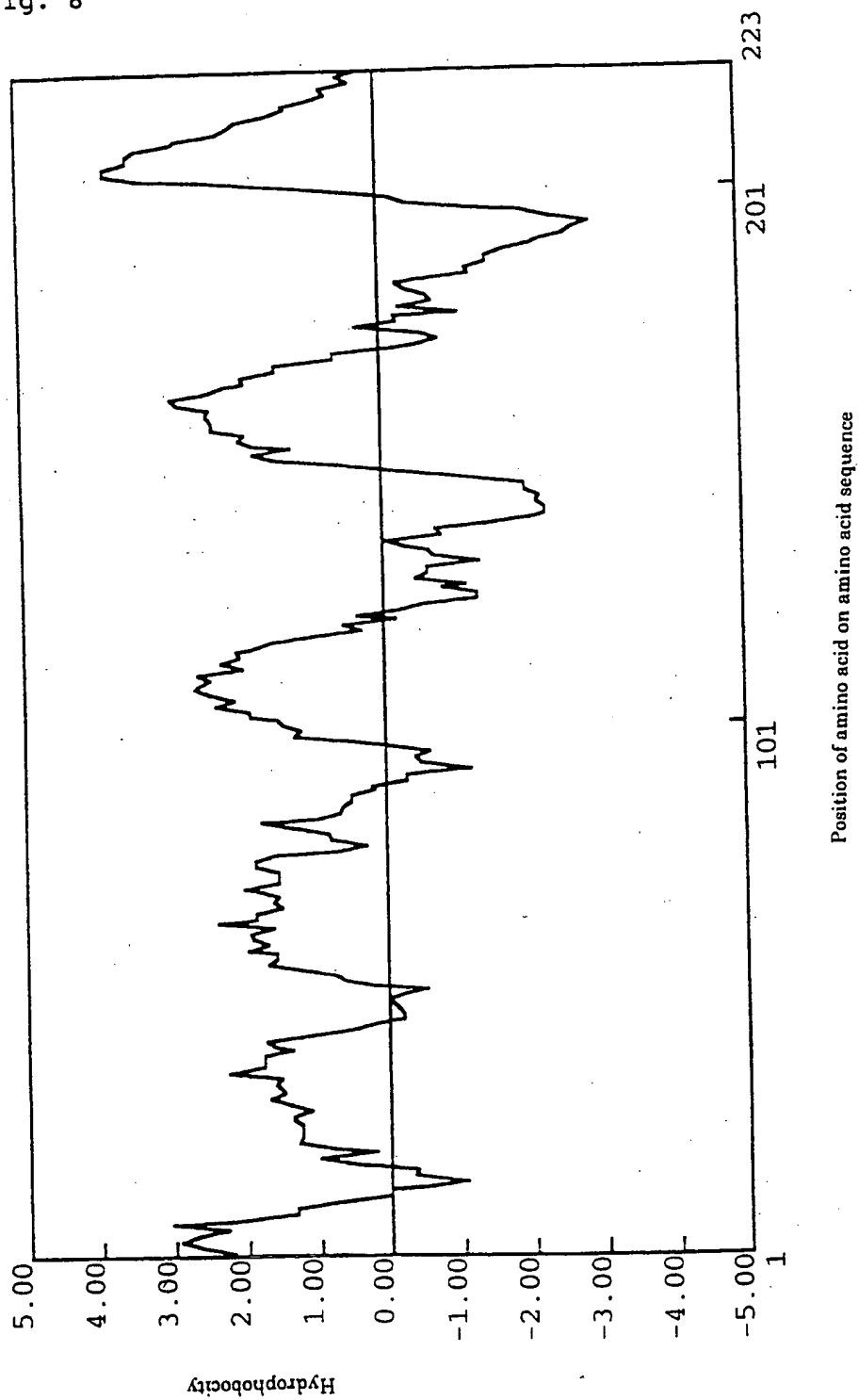
7/61

Fig. 7

p19P2	1	10	20	30	40	50	50
pg3-2/pg1-10	1	VGWVGNVLLV	LVIARVRRLLH	NVTNFIIGNL	ALSDVLMCTA	CVPLTLAYAF	50
	1	VGWVGNVLLV	LVIARVRRLLY	NVTNFIIGNL	ALSDVLMCTA	CVPLTLAYAF	50
p19P2	51	60	70	80	90	100	100
pg3-2/pg1-10	51	EPRGVVFGGG	LCHLVFFLQIP	VTVVVSFTL	TTIAVDRYVV	LVHPLRRRI-	100
	51	EPRGVVFGGG	LCHLVFFLQA	VTVVVSFTL	TTIAVDRYVV	LVHPLRRRI	100
p19P2	101	110	120	130	140	150	150
pg3-2/pg1-10	101	LRLSAYAVLA	IWL SAVIAL	PAAVHTYHVE	LKPHDVRLCE	EFWGSQERQR	150
p19P2	151	160	170	180	190	200	200
pg3-2/pg1-10	151	GLLV	TYLLPLLVITL	LSYVRVSVKL	RNRVVFGCVT	QSQADMDRAR	200
	151	QLYAWGLLV	TYLLPLLVITL	LSYVRVSVKL	RNRVVFGCVT	QSQADMDRAR	200
p19P2	201	210	220	230	240	250	250
pg3-2/pg1-10	201	RRRTFCLLVV	VWVFAICML	PVY.....	.....	.....	250
	201	RRRTFCLLVV	VWVFTLCML	PFF.....	.....	.....	250

8/61

Fig. 8



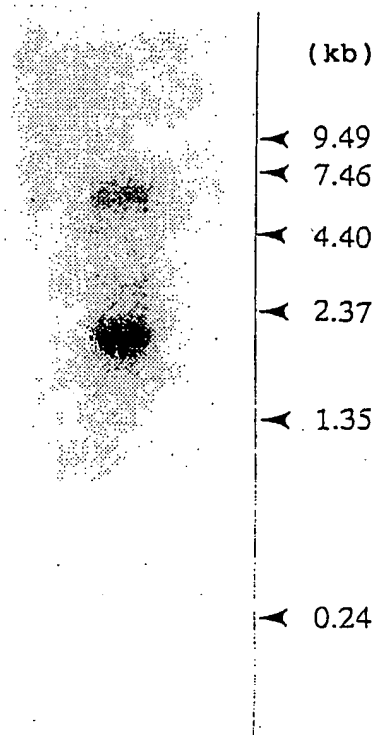
9/61

Fig. 9

1	CATCGTCAAGCAGATGAAGATCATCCACGAGGATGGCTACTCCGAGGGCCAGCAGAAATT	60
1		1
61	CTGCCCCCTTCTTCCCGGAGTGTCTTCCCGCTCTCCAAACCCCACTCCCAGGTGGCCATG	120
1	Met	1
121	GCCTCATCGACCACTCGGGGCCCCAGGGTTTCTGACTTATTTCTGGGCTGCCGCCGGCG	180
2	AlaSerSerThrThrArgGlyProArgValSerAspLeuPheSerGlyLeuProProAla	21
181	GTCACAACTCCCGCCAACCAGAGCGCAGAGGCCTCGGCGGGCAACGGGTGGTGGCTGGC	240
22	ValThrThrProAlaAsnGlnSerAlaGluAlaSerAlaGlyAsnGlySerValAlaGly	41
241	GCGGACGCTCCAGCCGTACGCCCTTCCAGAGCCTGCAGCTGGTGCATCAGCTGAAGGGG	300
42	AlaAspAlaProAlaValThrProPheGlnSerLeuGlnLeuValHisGlnLeuLysGly	61
301	CTGATCGTGCTGCTCTACAGCGTCGTGGTGGTGGTGGGGCTGGTGGGCAACTGCCCTGCTG	360
62	LeuIleValLeuLeuTyrSerValValValValValGlyLeuValGlyAsnCysLeuLeu	81
361	GTGCTGGTGATCGCGCGGTGCGCCGGCTGCACACGTGACGAACTTCCTCATCGGCAAC	420
92	ValLeuValIleAlaArgValArgArgLeuHisAsnValThrAsnPheLeuIleGlyAsn	101
421	CTGGCCTTGTCGACGTGCTCATGTGCACCGCCTGCGTGGCGCTCACGCTGGCCTATGCC	480
102	LeuAlaLeuSerAspValLeuMetCysThrAlaCysValProLeuThrLeuAlaTyrAla	121
481	TTGAGCCACGGGCTGGGTGTTCCGGCGGCGCCTGTGCCACCTGGTCTTCTTCTGTCAG	540
122	PheGluProArgGlyTrpValPheGlyGlyGlyLeuCysHisLeuValPhePheLeuGln	141
541	CCGGTCACCGTCTATGTGTCGGTGTTCACGCTCACCACCATCGCAGTGGACCGCTACGTC	600
142	ProValThrValTyrValSerValPheThrLeuThrThrIleAlaValAspArgTyrVal	161
501	GTGCTGGTGCACCCGCTGAGGCGGCGCATCTCGCTGCGCCTCAGCGCCTACGCTGTGCTG	660
162	ValLeuValHisProLeuArgArgArgIleSerLeuArgLeuSerAlaTyrAlaValLeu	181
561	GCCATCTGGGCGCTGTCCGCGGTGCTGGCGCTGCCCCCGCCCGTGCACACCTATCAGTG	720
182	AlaIleTrpAlaLeuSerAlaValLeuAlaLeuProAlaAlaValHisThrTyrHisVal	201
721	GAGCTCAAGCCGACGACGTGCGCCTCTGCGAGGAGTTCTGGGGCTCCAGGAGCGCCAG	780
202	GluLeuLysProHisAspValArgLeuCysGluGluPheTrpGlySerGlnGluArgGln	221
781	CGCCAGCTCTACGCTGGGGCTGCTGCTGGTCACTACCTGCTCCCTCTGCTGGTCACTC	840
222	ArgGlnLeuTyrAlaTrpGlyLeuLeuLeuValThrTyrLeuLeuProLeuLeuValIle	241
841	CTCCTGTCTTACGTCCGGGTGTCACTGAAGCTCCGCAACCGGTGGTCCCGGCTGCGTG	900
242	LeuLeuSerTyrValArgValSerValLysLeuArgAsnArgValValProGlyCysVal	261
901	ACCCAGAGCCAGGCGGACTGGGACCGCGCTCGGCGCGGCGCACCTTCTGCTTGCTGGTG	960
262	ThrGlnSerGlnAlaAspTrpAspArgAlaArgArgArgThrPheCysLeuLeuVal	281
961	GTGGTCGTGGTGGTGTTCGCGCTGCTGGCTGCGCTGCAGCTCTCAACCTGCTGCGG	1020
282	ValValValValValPheAlaValCysTrpLeuProLeuHisValPheAsnLeuLeuArg	301
1021	GACCTCGACCCCCACGCCATCGACCCTTACGCCTTTGGGCTGGTGCAGCTGCTCTGCCAC	1080
302	AspLeuAspProHisAlaIleAspProTyrAlaPheGlyLeuValGlnLeuLeuCysHis	321
1081	TGGCTCGCCATGAGTTTCGGCTGTCTACAACCCCTTCATCTACGCCTGGCTGCAGCAGC	1140
322	TrpLeuAlaMetSerSerAlaCysTyrAsnProPheIleTyrAlaTrpLeuHisAspSer	341
1141	TTCCGCGAGGAGCTCGGCAAACTGTTGGTTCGCTTGGCCCCGCAAGATAGCCCCCATGGC	1200
342	PheArgGluGluLeuArgLysLeuLeuValAlaTrpProArgLysIleAlaProHisGly	361
1201	CAGAATATGACCGTCAGCGTGGTCACTCTGATGCCACTTAGCCAGGCCTTGGTCAAGGAGC	1260
362	GlnAsnMetThrValSerValValIle***	371
1261	TCCACTTCAACTGGCCTCCTAGGGCACCCTCGAGGTCAATCTGGTGTCTATTCTCAGCA	1320
		371
1321	CCAGAGCTAGC	1331
		371

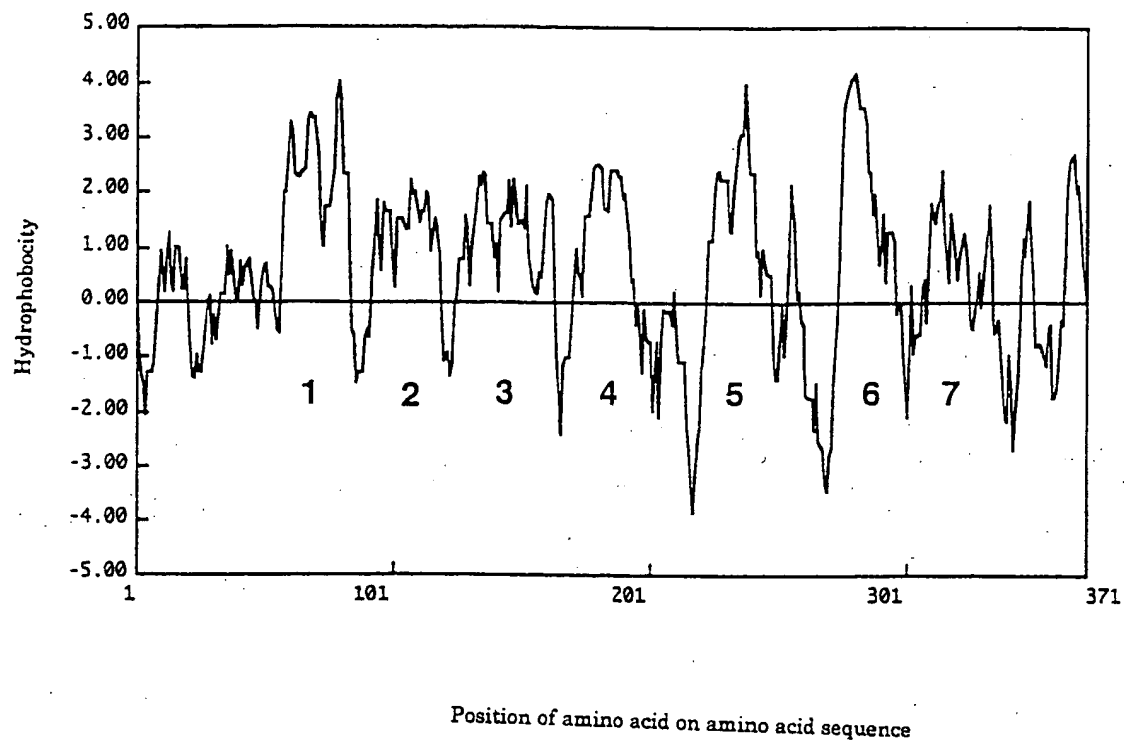
10/61

Fig. 10



11/61

Fig. 11





12/61

Fig. 12

5'	9			18			27			36			45			54		
	CTG	TGT	GTC	ATC	GCG	GTG	GAT	AGG	TAC	GTG	GTT	CTG	GTG	CAC	CCG	CTA	CGT	CGG
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	Leu	Cys	Val	Ile	Ala	Val	Asp	Arg	Tyr	Val	Val	Leu	Val	His	Pro	Leu	Arg	Arg
	63			72			81			90			99			108		
	CGC	ATT	TCA	CTG	AGG	CTC	AGC	GCC	TAC	GCG	GTG	CTG	GGC	ATC	TGG	GCT	CTA	TCT
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	Arg	Ile	Ser	Leu	Arg	Leu	Ser	Ala	Tyr	Ala	Val	Leu	Gly	Ile	Trp	Ala	Leu	Ser
	117			126			135			144			153			162		
	GCA	GTG	CTG	GCG	CTG	CCG	GCC	GCG	GTG	CAC	ACC	TAC	CAT	GTG	GAG	CTC	AAG	CCC
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	Ala	Val	Leu	Ala	Leu	Pro	Ala	Ala	Val	His	Thr	Tyr	His	Val	Glu	Leu	Lys	Pro
	171			180			189			198			207			216		
	CAC	GAC	GTG	AGC	CTC	TGC	GAG	GAG	TTC	TGG	GGC	TCG	CAG	GAG	CGC	CAA	CGC	CAG
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	His	Asp	Val	Ser	Leu	Cys	Glu	Glu	Phe	Trp	Gly	Ser	Gln	Glu	Arg	Gln	Arg	Gln
	225			234			243			252			261			270		
	ATC	TAC	GCC	TGG	GGG	CTG	CTT	CTG	GCG	ACC	TAT	TTG	CTC	CCC	CTG	CTG	GCC	ATC
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	Ile	Tyr	Ala	Trp	Gly	Leu	Leu	Leu	Gly	Thr	Tyr	Leu	Leu	Pro	Leu	Leu	Ala	Ile
	279			288			297			306			315			324		
	CTC	CTG	TCT	TAC	GTA	CGG	GTG	TCA	GTG	AAG	CTG	AGG	AAC	CGC	GTG	GTG	CCT	GGC
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	Leu	Leu	Ser	Tyr	Val	Arg	Val	Ser	Val	Lys	Leu	Arg	Asn	Arg	Val	Val	Pro	Gly
	333			342			351			360			369			378		
	AGC	GTG	ACC	CAG	AGT	CAA	GCT	GAC	TGG	GAC	CGA	GCG	CGT	CGC	CGC	CGC	ACT	TTC
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	Ser	Val	Thr	Gln	Ser	Gln	Ala	Asp	Trp	Asp	Arg	Ala	Arg	Arg	Arg	Arg	Thr	Phe
	387			396			405			414			423			432		
	TGT	CTG	CTG	GTG	GTG	GTG	GTA	GTG	TTC	ACG	CTC	TGC	TGG	CTG	CCC	TTC	TAC	
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	Cys	Leu	Leu	Val	Val	Val	Val	Val	Phe	Thr	Leu	Cys	Trp	Leu	Pro	Phe	Tyr	

CT 3'

--

—

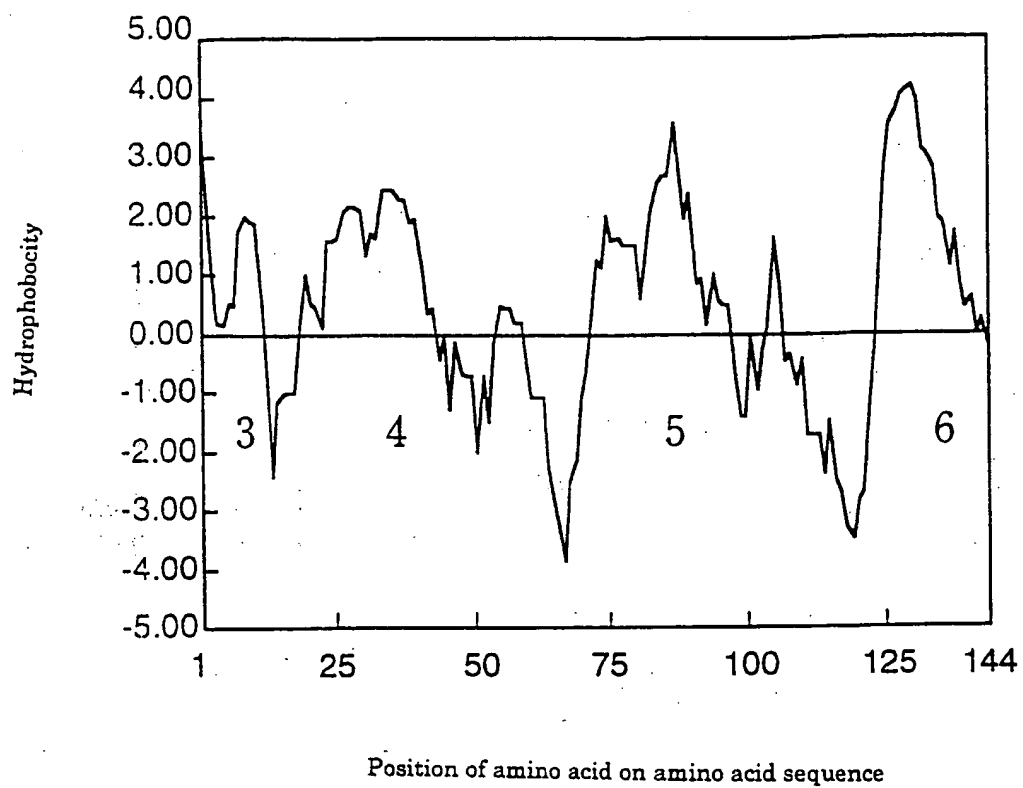
13/61

Fig. 13

p19P2	1	10	20	30	40	50	50
pG3-2/pG1-10	1	VGMVGNLLV	LVIARVRRLLH	NVTNFLICNL	ALSDVLMCTA	CVPLTLAYAF	50
p5S38	-79	VGMVGNLLV	LVIARVRRLLY	NVTNFLICNL	ALSDVLMCTA	CVPLTLAYAF	50
							-30
p19P2	51	60	70	80	90	100	100
pG3-2/pG1-10	51	EPRGWFVGGG	LCHLVFFLOP	VTVVVSFTL	TTIAVDRVVV	LVIHPLRRRI	100
p5S38	-29	EPRGWFVGGG	LCHLVFFLOA	VTVVVSFTL	TTIAVDRVVV	LVIHPLRRRI	100
					CVIAVDRVVV	LVIHPLRRRI	21
p19P2	101	110	120	130	140	150	150
pG3-2/pG1-10	101	LRLSAYAVLA	IWL SAVLAL	PAAVHTYHVE	LKPHDVRLCE	EFVGSQERQR	150
p5S38	22	LRLSAYAVLG	IWA SAVLAL	PAAVHTYHVE	LKPHDVSLCE	EFVGSQERQR	71
p19P2	151	160	170	180	190	200	200
pG3-2/pG1-10	151	GLLLV	TYLLPLLVL	LSYVRVSVKL	RNRVVEGCVT	QSQADIDRAR	200
p5S38	72	QIYAMGLLLG	TYLLPLLVL	LSYVRVSVKL	RNRVVEGCVT	QSQADIDRAR	121
					RNRVVEGCVT	QSQADIDRAR	
p19P2	201	210	220	230	240	250	250
pG3-2/pG1-10	201	RRRTFCLLVV	VVVVFAICWL	PPV			250
p5S38	122	RRRTFCLLVV	VVVVFTLCWL	PPV			171

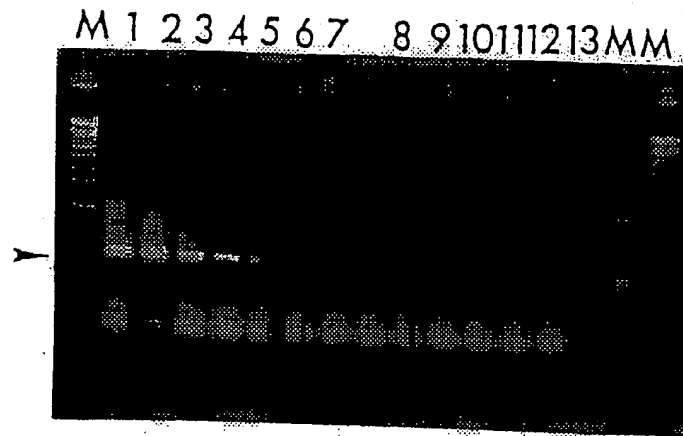
14/61

Fig. 14



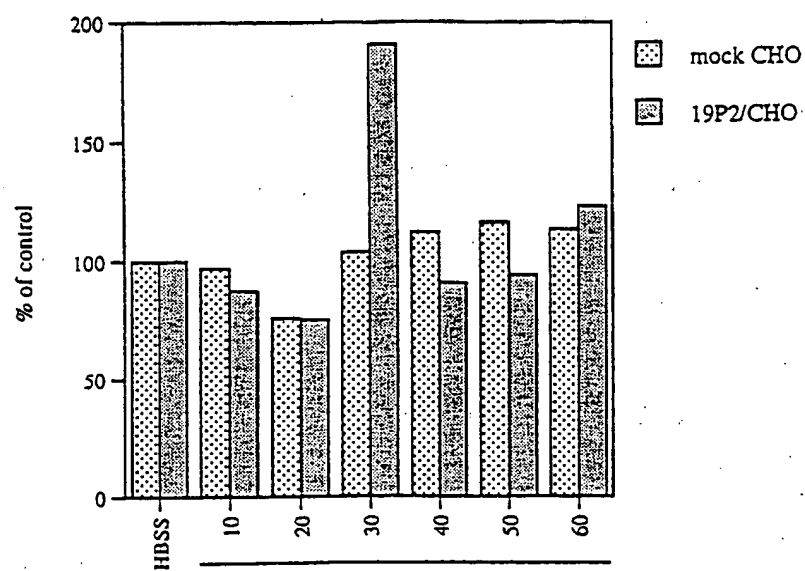
15/61

Fig. 15



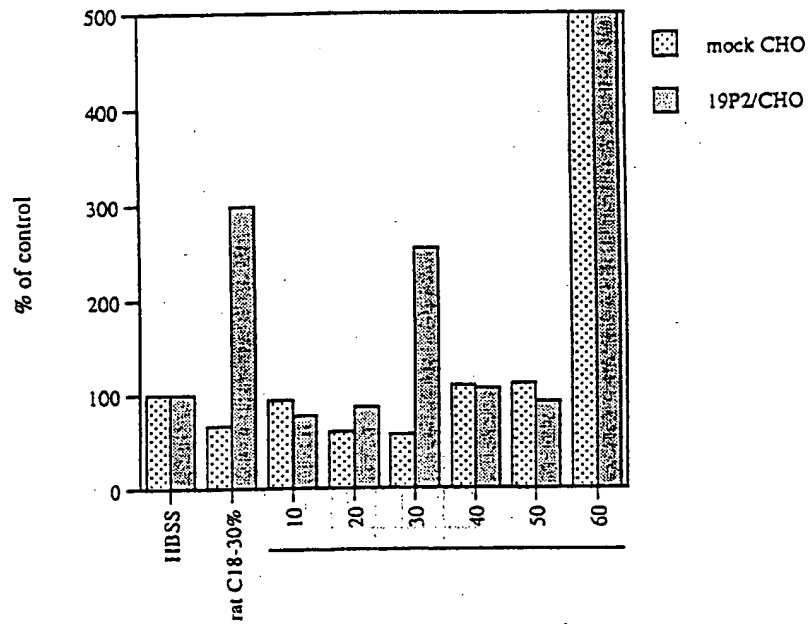
16/61

Fig. 16



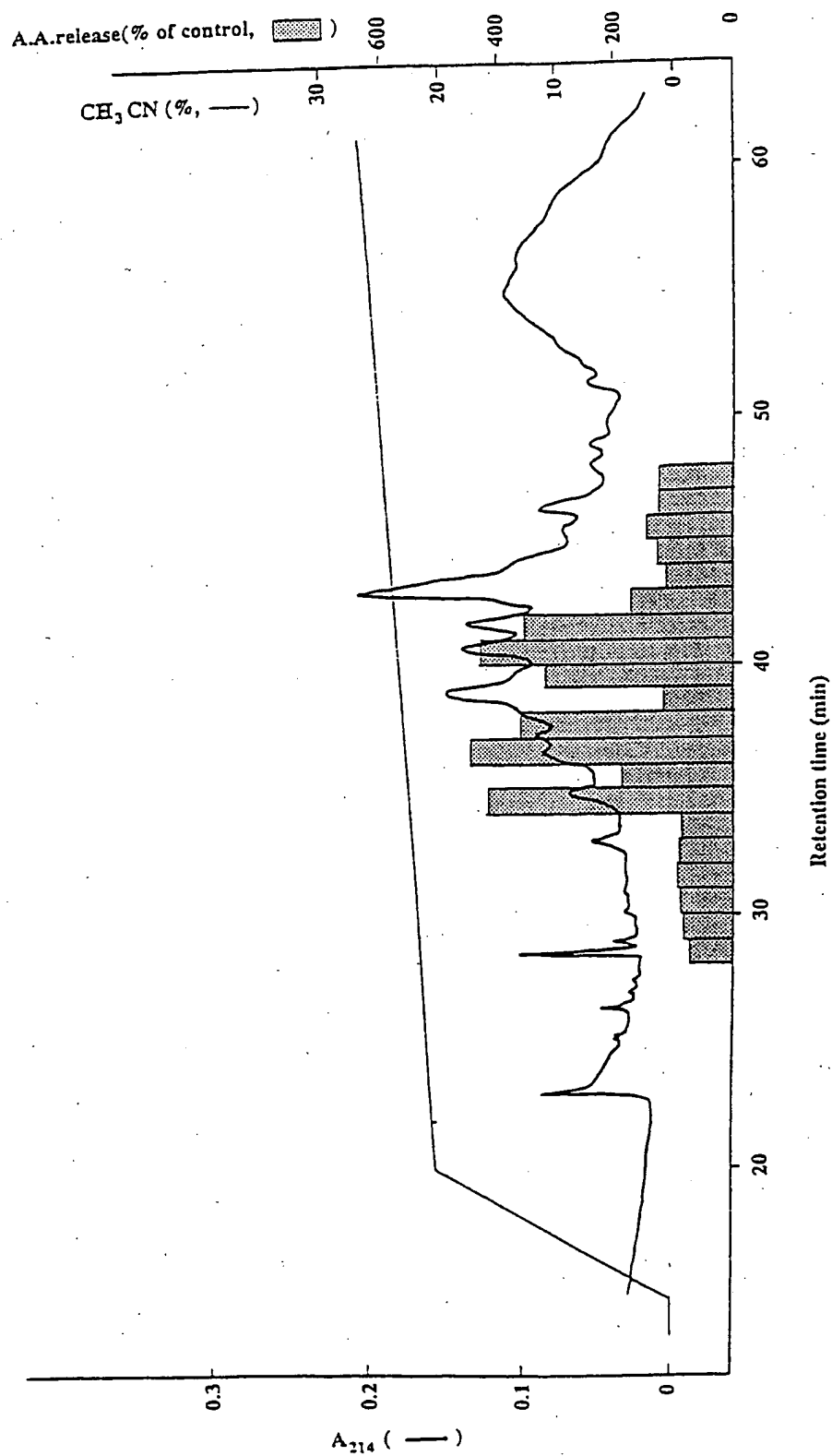
17/61

Fig. 17



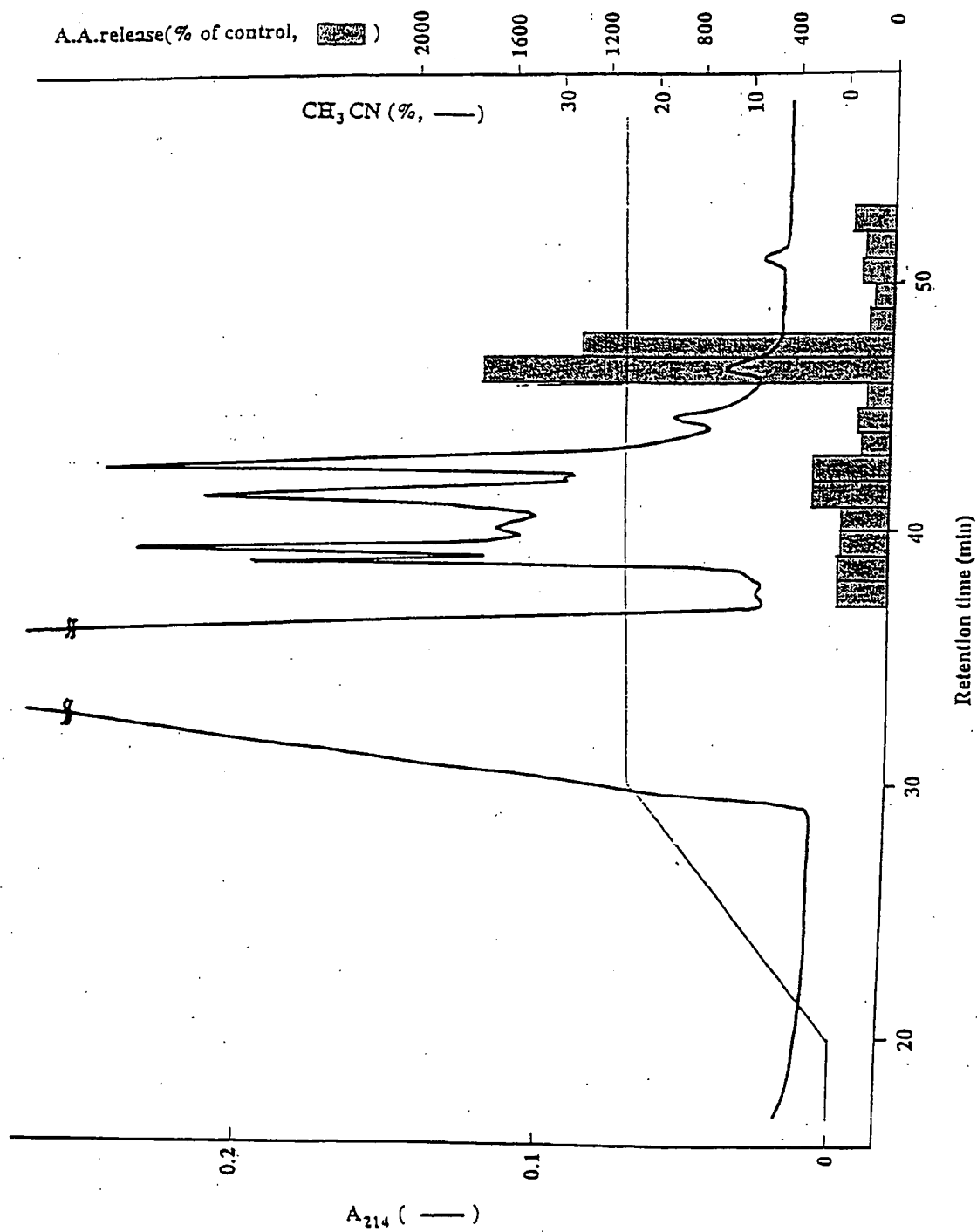
18/61

Fig. 18



19/61

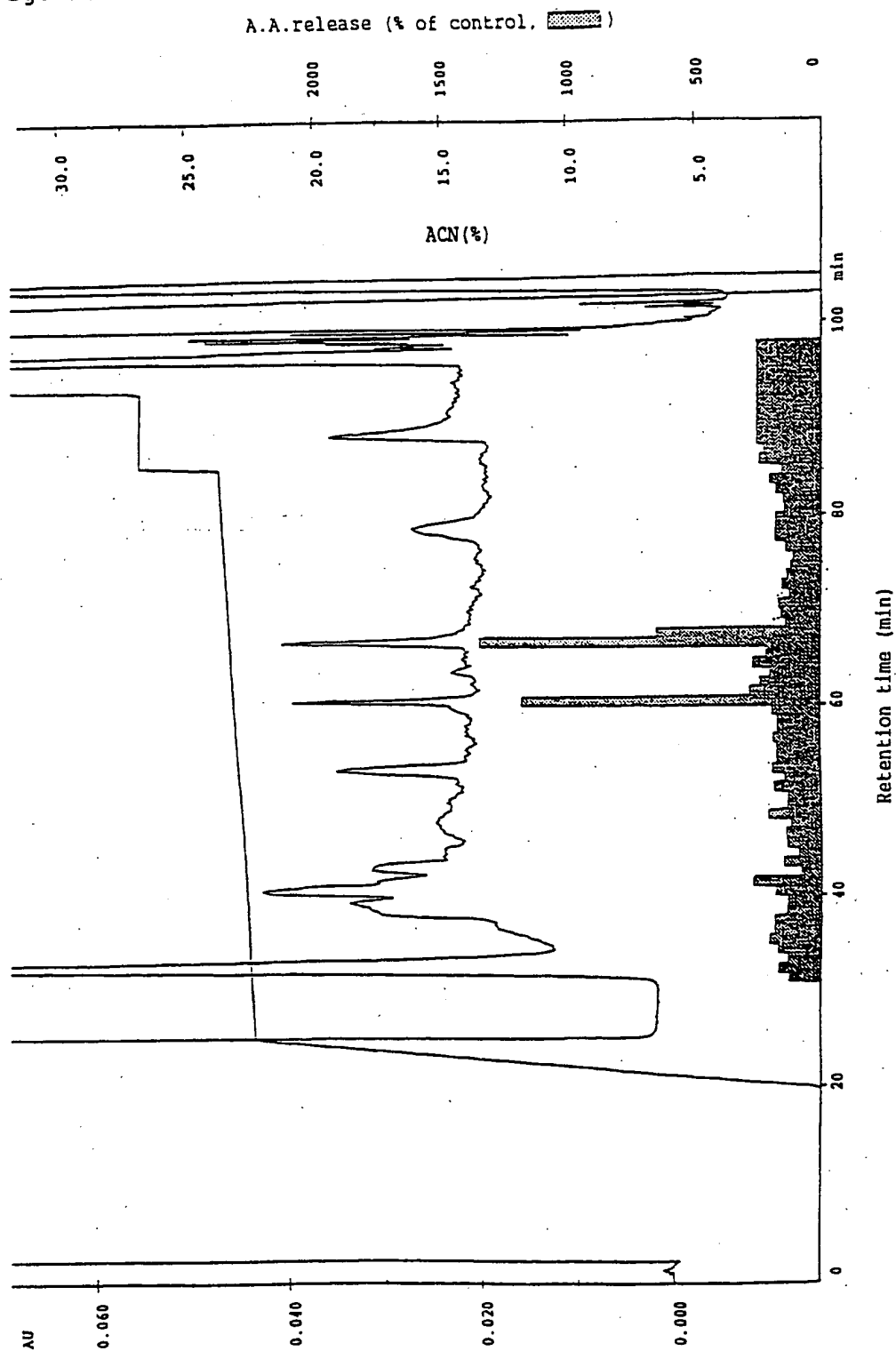
Fig. 19





20/61

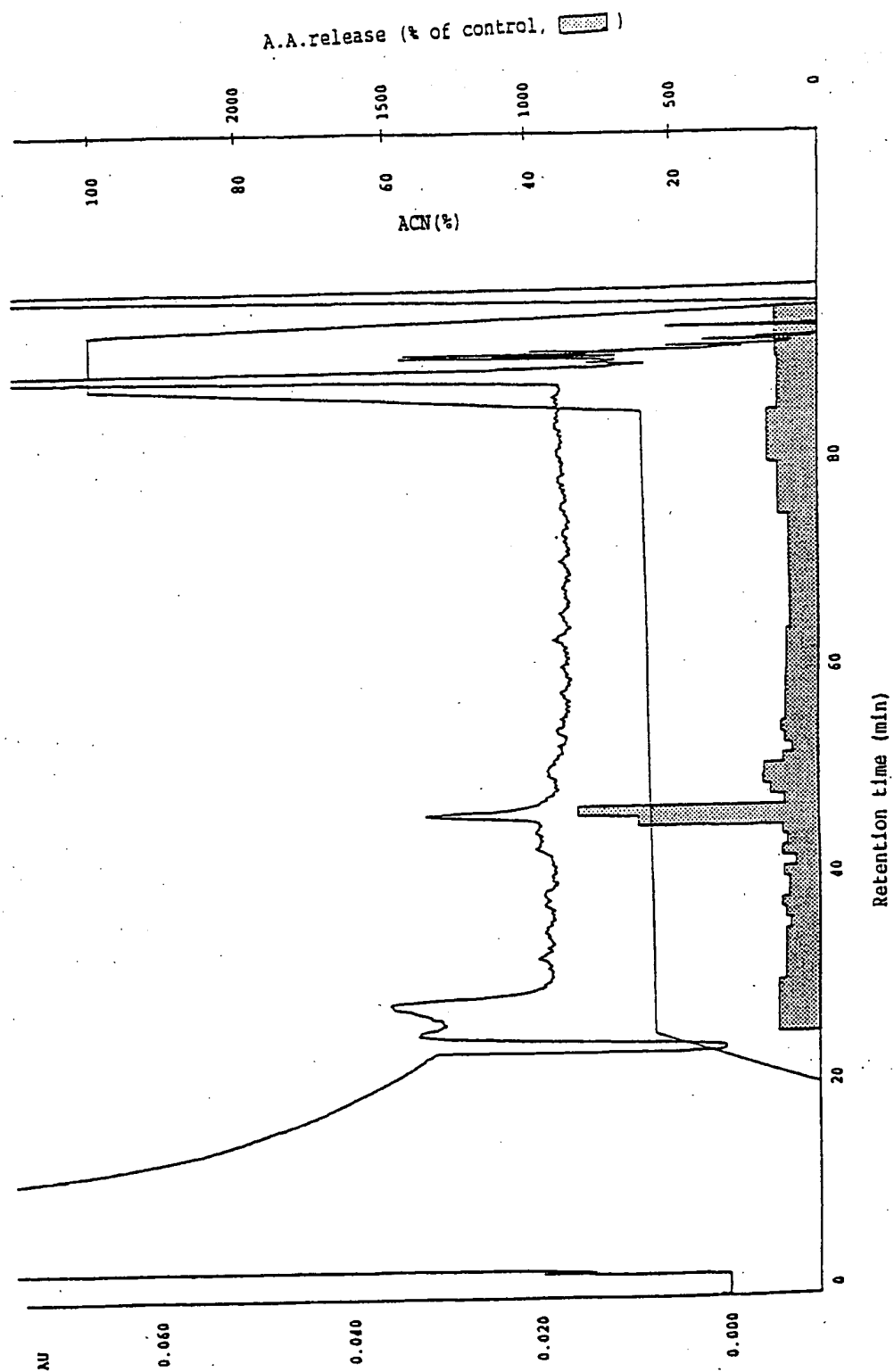
Fig. 20



A215

21/61

Fig. 21



A215

22/61

Fig. 22

P5-1

9 18 27 36 45 54

5' GCC CAC CAG CAC TCC ATG GAG ATC CGC ACC CCC GAC ATC AAC CCT GCC TGG TAC  
-----  
Ala His Gln His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn Pro Ala Trp Tyr

63 72

GCG GGC CGT GGG ATC CGG CCC G 3'

-----  
Ala Gly Arg Gly Ile Arg Pro

P3-2

```

1  GTGGAATGAAGGCGGTGGGGGCCCTGGCTCCTCTGCCTGCTGCTGGGCCTGGCCCTG      59
1      MetLysAlaValGlyAlaTrpLeuLeuCysLeuLeuLeuLeuGlyLeuAlaLeu      18
60 CAGGGGGCTGCCAGCAGAGCCCACCAGCACTCCATGGAGATCCGCACCCCCGACATCAAC      119
19  GlnGlyAlaAlaSerArgAlaHisGlnHisSerMetGluIleArgThrProAspIleAsn      38
                                     ←————— PDN —————→
120 CCTGCCT      126
39  ProAla      40

```

24/61

Fig. 24(a)

1	GTGGAATGAAGGCGGTGGGGGCTGGCTCCTCTGCCTGCTGCTGCTGGGCCTGGCCCTG	59
1	MetLysAlaValGlyAlaTrpLeuLeuCysLeuLeuLeuLeuGlyLeuAlaLeu	18
60	CAGGGGGCTGCCAGCAGAGCCCACCAGCACTCCATGGAGATCCGCACCCCCGACATCAAC	119
19	GlnGlyAlaAlaSerArgAlaHisGlnHisSerMetGluIleArgThrProAspIleAsn	38
120	CCTGCCTGGTACGCRGGCCGTGGGATCCGGCCCGTGGGCCTTCGGCCGGCGAAGAGCT	179
39	ProAlaTrpTyrAlaGlyArgGlyIleArgProValGlyArgPheGlyArgArgAla	58
180	GGCCCGGGGGACGGACCCAGGCCTGGCCCCGGCGTGTGCCGGCCTGCTTCCGCCTGGAA	239
59	AlaProGlyAspGlyProArgProGlyProArgArgValProAlaCysPheArgLeuGlu	78
240	GGCGGYCTGAGCCCTCCCGAGCCCTCCCGGGGCGGCTGACGGCCAGCTGGTCCAGGAA	299
79	GlyGlyAlaGluProSerArgAlaLeuProGlyArgLeuThrAlaGlnLeuValGlnGlu	98
300	TAACAGCGGGAGCCTGCCCCCACCCTCCTCCTCCACCAGCCACCTTCCCTCCAGTCCT	359
98		98
360	AATAAAGCAGCTGGCTTGTT	380
98		98

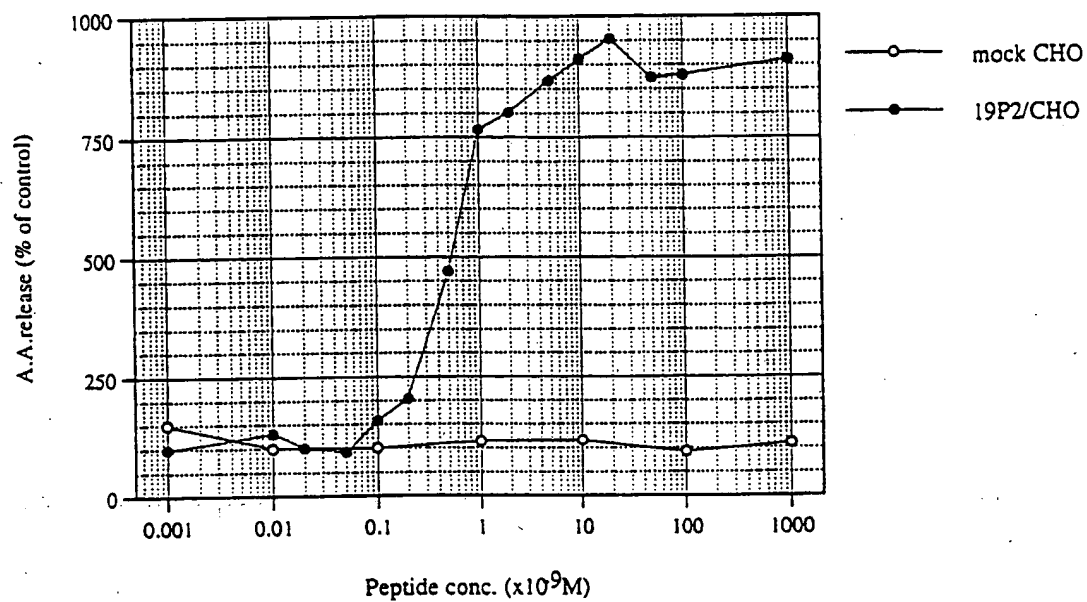
25/61

Fig. 24(b)

1	GTGGAATGAAGCGGTGGGGGCTGGCTCCTCTGCCTGCTGCTGCTGGGCCTGGCCCTG	59
1	MetLysAlaValGlyAlaTrpLeuLeuCysLeuLeuLeuGlyLeuAlaLeu	18
60	CAGGGGGCTGCCAGCAGAGCCCACCAGCACTCCATGGAGATCCGCACCCCCGACATCAAC	119
19	GlnGlyAlaAlaSerArgAlaHisGlnHisSerMetGluIleArgThrProAspIleAsn	38
120	CCTGCCTGGTACGCRGGCCGTGGGATCCGGCCCCGTGGGCGCTTCGGCCGGCGAAGAGCT	179
39	ProAlaTrpTyrAlaGlyArgGlyIleArgProValGlyArgPheGlyArgArgArgAla	58
180	GCCCTGGGGGACGGACCCAGGCCTGGCCCCGGCGTGTGCCGGCCTGCTTCCGCCTGGAA	239
59	AlaLeuGlyAspGlyProArgProGlyProArgArgValProAlaCysPheArgLeuGlu	78
240	GGCGGYGCTGAGCCCTCCCGAGCCCTCCCGGGGCGGCTGACGGCCCAGCTGGTCCAGGAA	299
79	GlyGlyAlaGluProSerArgAlaLeuProGlyArgLeuThrAlaGlnLeuValGlnGlu	98
300	TAACAGCGGGAGCCTGCCCCCACCCTCCTCCTCCACCAGCCACCTTCCCTCCAGTCCT	359
98		98
360	AATAAAAGCAGCTGGCTTGTT	380
98		98

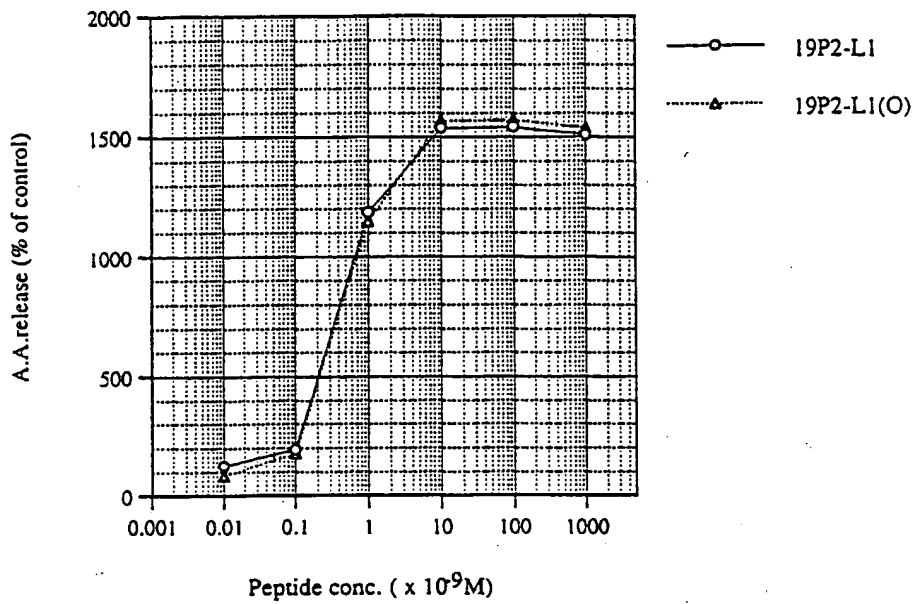
26/61

Fig. 25



27/61

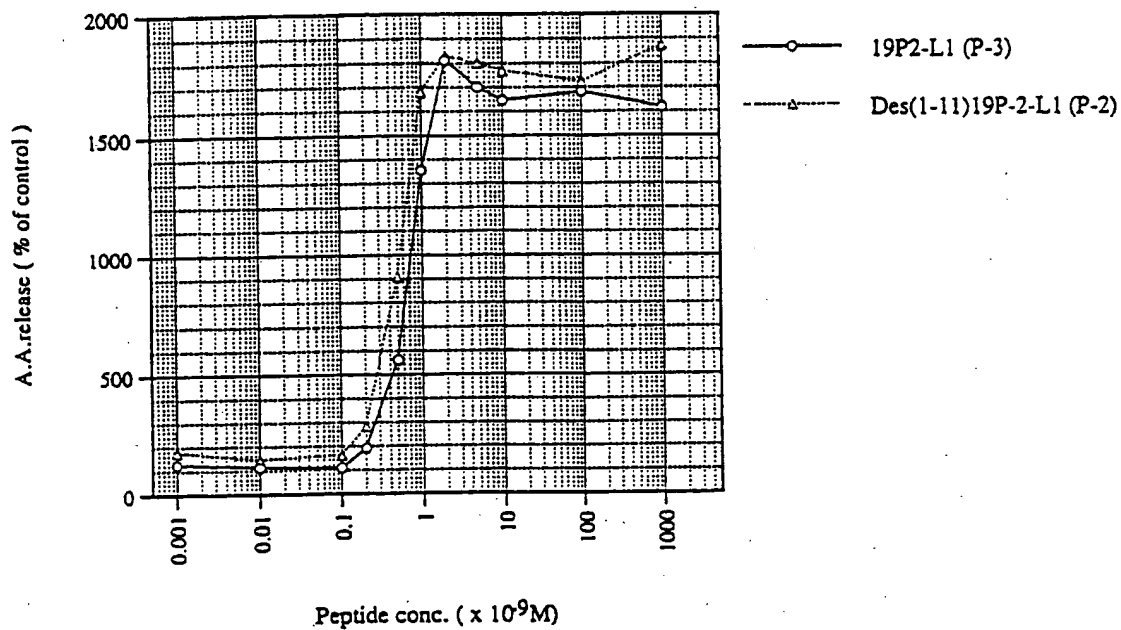
Fig. 26





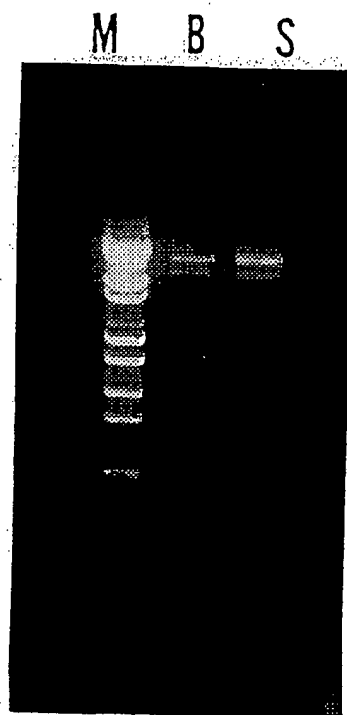
28/61

Fig. 27



29/61

Fig. 28



30/61

Fig. 29

10	20	30	40	50	60
ATGAAGGCGG	TGGGGGCCTG	GCTCCTCTGC	CTGCTGCTGC	TGGGCCTGGC	CCTGCAGGGG
70	80	90	100	110	120
GCTGCCAGCA	GAGCCCAcca	GCACTCCATG	GAGATCCGCA	GTGAGTGTCT	AGCCCCGCCC
130	140	150	160	170	180
CTGCCCCCAG	GGGTCACAGG	GGGGGCCTGG	CCACTTCCTG	GGCTGGGACA	TCCTTGCTAA
190	200	210	220	230	240
GCATCCTGGG	GTTGGGGTTT	GGCCTCCTGT	TCCCCAGACC	CTTCCCCCAG	GTGGCCCCGA
250	260	270	280	290	300
CAGGTGCTCC	CAAGGGTCCC	GGCCCAGCAC	ACGGGGGAGG	GTCACTCCTC	ACCACACGGG
310	320	330	340	350	360
TGGCCTGGGG	CTGAGTGCAC	GTCACCCATG	AGAACGGGGC	TGTGAGGACA	GGAAAGGAAG
370	380	390	400	410	420
GGGAGTGTGT	CCTGGTGTGA	GTCTGAAATC	CTACTTCCCA	AAGCCACCCC	AGCACCAGAA
430	440	450	460	470	480
ATGGGCGCTC	CGGGTGAACC	TCCTGTGCGG	GTGGGTGGTC	CTGGCATGGC	CTGGGCGACA
490	500	510	520	530	540
GGCAGCCATG	AGCTGAGCAC	ACACCCGGCC	CGGCCACCAG	GGCTGTATGC	TCCAGGGCAC
550	560	570	580	590	600
AGGCCTCCAT	GCGCTCTTCT	CTCTCTTTCC	AGCCCCCGAC	ATCAACCCTG	CCTGGTACGC
610	620	630	640	650	660
AGGCCGTGGG	ATCCGGCCCCG	TGGGCCGCTT	CGGCCGGCGA	AGAGCTGCCC	TGGGGGACGG
670	680	690	700	710	720
ACCCAGGCCT	GGCCCCCGGC	GTGTGCCGGC	CTGCTTCCGC	CTGGAAGGCG	GTGCTGAGCC
730	740	750	760	770	780
CTCCCGAGCC	CTCCCGGGGC	GGCTGACGGC	CCAGCTGGTC	CAGGAATAA	.....

31/61

Fig. 30

genome	10	20	30	40	50	
cDNA	1 ATGAAGGCGG	TGGGGGCGCTG	GCTCCTCTGC	CTGCTGCTGC	TGGGCCTGGC	50
	1 ATGAAGGCGG	TGGGGGCGCTG	GCTCCTCTGC	CTGCTGCTGC	TGGGCCTGGC	50
genome	60	70	80	90	100	
cDNA	51 CCTGCAGGGG	GCTGCCAGCA	GAGCCACCA	GCACTCCATG	GAGATCCGCA	100
	51 CCTGCAGGGG	GCTGCCAGCA	GAGCCACCA	GCACTCCATG	GAGATCCGCA	100
genome	110	120	130	140	150	
cDNA	101 GTGAGTGTCT	AGCCCCGCCC	CTGCCCCCAG	GGGTACAGG	GGGGGCGCTGG	150
	101 -----	-----	-----	-----	-----	150
genome	160	170	180	190	200	
cDNA	151 CCACTTCCTG	GGCTGGGACA	TCCTTGCTAA	GCACTCCTGGG	GTTGGGGTTT	200
	151 -----	-----	-----	-----	-----	200
genome	210	220	230	240	250	
cDNA	201 GGCCTCCTGT	TCCCCAGACC	CTTCCCCCAG	GTGGCCCGGA	CAGGTGCTCC	250
	201 -----	-----	-----	-----	-----	250
genome	260	270	280	290	300	
cDNA	251 CAAGGTCC	GGCCAGCAC	ACGGGGGAGG	GTCACCTCTC	ACCACACGGG	300
	251 -----	-----	-----	-----	-----	300
genome	310	320	330	340	350	
cDNA	301 TGGCCTGGGG	CTGAGTGCAC	GTCACCCATG	AGAACGGGGC	TGTGAGGACA	350
	301 -----	-----	-----	-----	-----	350
genome	360	370	380	390	400	
cDNA	351 GGAAAGGAAG	GGGAGTGTGT	CCTGGTGTGA	GTCTGAAATC	CTACTTCCCA	400
	351 -----	-----	-----	-----	-----	400
genome	410	420	430	440	450	
cDNA	401 AAGCCACCCC	AGCACCAGAA	ATGGGCGCTC	CGGGTGAACC	TCCTGTGCGG	450
	401 -----	-----	-----	-----	-----	450
genome	460	470	480	490	500	
cDNA	451 GTGGGTGGTC	CTGGCATGGC	CTGGGCGACA	GGCAGCCATG	AGCTGAGCAC	500
	451 -----	-----	-----	-----	-----	500
genome	510	520	530	540	550	
cDNA	501 ACACCCGGCC	CGGCCACCAG	GGCTGTATGC	TCCAGGGCAC	AGGCCTCCAT	550
	501 -----	-----	-----	-----	-----	550
genome	560	570	580	590	600	
cDNA	551 GCGCTCTTCT	CTCTCTTTCC	AGCCCCGAC	ATCAACCCTG	CCTGGTACGC	600
	551 -----	-----	---CCCCGAC	ATCAACCCTG	CCTGGTACGC	600
genome	610	620	630	640	650	
cDNA	601 AGGCCGTGGG	ATCCGGCCCCG	TGGGCGGCTT	CGGCCGGCGA	AGAGCTGCCC	650
	601 GGGCCGTGGG	ATCCGGCCCCG	TGGGCGGCTT	CGGCCGGCGA	AGAGCTGCCC	650
genome	660	670	680	690	700	
cDNA	651 TGGGGGACGG	ACCCAGGCCT	GGCCCCCGGC	GTGTGCCGGC	CTGCTTCCGC	700
	651 CGGGGGACGG	ACCCAGGCCT	GGCCCCCGGC	GTGTGCCGGC	CTGCTTCCGC	700
genome	710	720	730	740	750	
cDNA	701 CTGGAAGGCG	GTGCTGAGCC	CTCCCGAGCC	CTCCCGGGGC	GGCTGACGGC	750
	701 CTGGAAGGCG	GCGCTGAGCC	CTCCCGAGCC	CTCCCGGGGC	GGCTGACGGC	750
genome	760	770	780	790	800	
cDNA	751 CCAGCTGGTC	CAGGAATAA.	.....	.....	.....	800
	751 CCAGCTGGTC	CAGGAATAA.	.....	.....	.....	800



33/61

Fig. 32

1	GGCATCATCCAGGAAGACGGAGCATGGCCCTGAAGACGTGGCTTCTGTGCTTGCTGCTG	59
1	MetAlaLeuLysThrTrpLeuLeuCysLeuLeuLeu	12
60	CTAAGCTTGGTCCTCCCAGGGGCTTCCAGCCGAGCCACCAGCACTCCATGGAGACAAGA	119
13	LeuSerLeuValLeuProGlyAlaSerSerArgAlaHisGlnHisSerMetGluThrArg	32
120	ACCCCTGATATCAATCCTGCCTGGTACACGGGCGCGGGATCAGGCCTGTGGGCGGCTTC	179
33	ThrProAspIleAsnProAlaTrpTyrThrGlyArgGlyIleArgProValGlyArgPhe	52
180	GGCAGGAGAAGGGCAACCCCGAGGGATGTCACTGGACTTGGCCAACTCAGCTGCCTCCCA	239
53	GlyArgArgArgAlaThrProArgAspValThrGlyLeuGlyGlnLeuSerCysLeuPro	72
240	CTGGATGGACGCACCAAGTTCTCTCAGCGTGGATAACACCCCGAGCTCGAGAAGACAGTGC	299
73	LeuAspGlyArgThrLysPheSerGlnArgGly***	83
300	TGCTGAGCCCAAGCCCACACTCCCTGTCCCCTGCAGACCCTCCTCTACCCTCCCTCTCCT	359
83		83
360	CTGCT	364
83		83

Fig. 33

bovine.aa		M K A V G A W L L					
	10	20	30	40	50		
bovine.seq	-18	.....GT	GGAATGAAGG	CGGTGGGGGC	CTGGCTCCTC	32	
rat.seq	1	GGCATCATCC	AGGAAGACGG	AGCATG---G	CCCTGAAGAC	GTGGCTTCTG	50
bovine.aa		C L L L L G L A L Q G A A S R A H					
	60	70	80	90	100		
bovine.seq	33	TGCCTGCTGC	TGCTGGGCCCT	GCCCCTGCAG	GGGGCTGCCA	GCAGAGCCCCA	82
rat.seq	51	<u>TGCTTGCTGC</u>	TGCTAAGCTT	GGTCCTCCCA	GGGGCTTCCA	GCCGAGCCCCA	100
		R1					
bovine.aa		Q H S M E I R T P D I N P A W Y A					
	110	120	130	140	150		
bovine.seq	83	CCAGCACTCC	ATGGAGATCC	GCACCCCGCA	CATCAACCCT	GCCTGGTIACG	132
rat.seq	101	CCAGCACTCC	ATGGAGACAA	GAACCCCTGA	TATCAATCCT	<u>GCCTGGTIACA</u>	150
					R3		
bovine.aa		G R G I R P V G R F G R R R A A					
	160	170	180	190	200		
bovine.seq	133	CGGGCCGTGG	GATCCGGGCC	GTGGGCCGCT	TGGGCCGGGG	AAGAGCTGCC	182
rat.seq	151	<u>CGGGCCGCGG</u>	<u>GATCAGGCCT</u>	<u>GTGGGCCGCT</u>	<u>TGGGCAGGAG</u>	<u>AAGGGCAACC</u>	200
			R4				
bovine.aa		P G D G P R P G P R R V P A C F R					
	210	220	230	240	250		
bovine.seq	183	CCGGGGGACG	GACCCAGGCC	TGGCCCCCGG	CGTGTGCCCG	CCTGCTTCCG	232
rat.seq	201	CCGAGGGATG	TCACTGGACT	TGGC-----	---CAACTCA	GCTGCCTCCC	250
bovine.aa		L E G G A E P S R A L P G R L T A					
	260	270	280	290	300		
bovine.seq	213	CCTGGAAGGC	GGCGCTGAGC	CCTCCCGAGC	CCTCCCGGGG	CGGCTGACGG	282
rat.seq	251	ACTGGATGGA	CGCACCAAGT	TCTCTCAGCG	TGGATAACAC	CCCAGCTCGA	300
bovine.aa		Q L V Q E *					
	310	320	330	340	350		
bovine.seq	283	CCCAGCTGGT	CCAGGAATAA	CAGCGGGAGC	CTGCCCCCCA	CCCTCTCTCC	332
rat.seq	301	GAAGACAGTG	CTGCTGAGCC	CAAGCCCACA	CTCCCTGTCC	CCTGCAGACC	350
	360	370	380	390	400		
bovine.seq	333	TCCACCAGCC	ACCTTCCCTC	CAGTCTTAAT	AAAAGCAGCT	GGCTTGTT..	382
rat.seq	351	CTCCTCTACC	CTCCCTCTCC	TCTGCT....	.....	.....	400

Fig. 34

1	GGCCTCCTCGGAGGAGCCAAGGGATGAAGGTGCTGAGGGCCTGGCTCCTGTGCCTGCTG	59
1	MetLysValLeuArgAlaTrpLeuLeuCysLeuLeu	12
60	ATGCTGGGCCTGGCCCTGCGGGGAGCTGCAAGTCGTACCCATCGGCACTCCATGGAGATC	119
13	MetLeuGlyLeuAlaLeuArgGlyAlaAlaSerArgThrHisArgHisSerMetGluIle	32
120	CGCACCCCTGACATCAATCCTGCCTGGTACGCCAGTCGCGGGATCAGGCCTGTGGGCCGC	179
33	ArgThrProAspIleAsnProAlaTrpTyrAlaSerArgGlyIleArgProValGlyArg	52
180	TTCGGTCGGAGGAGGGCAACCCTGGGGGACGTCCCCAAGCCTGGCCTGCGACCCCGGCTG	239
53	PheGlyArgArgArgAlaThrLeuGlyAspValProLysProGlyLeuArgProArgLeu	72
240	ACCTGCTTCCCCCTGGAAGGCGGTGCTATGTCGTCCCAGGATGGCTGACAGCCAGCTTGT	299
73	ThrCysPheProLeuGluGlyGlyAlaMetSerSerGlnAspGly***	87
300	CAAGAACTCACTCTGGAGCCTCCCCCACCCACCCTCTCCTCTCCTTCGGGCTCCTTTC	359
87		87
360	CC	361
87		87



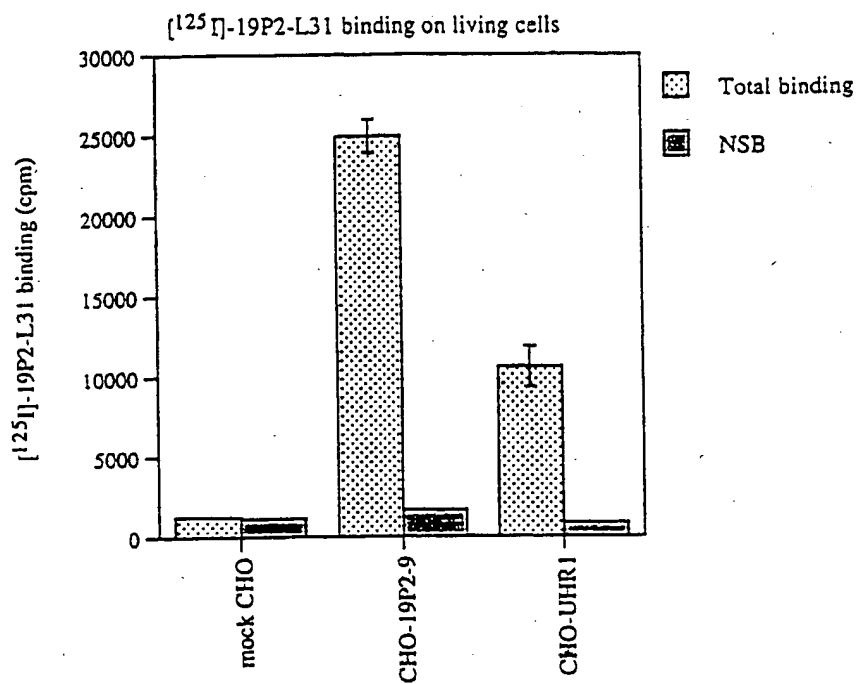
36/61

Fig. 35

	10	20	30	40	50	
bovine.aa	1 MKAVGAWLLC	LLLLGLALQG	AASRAHQHSM	EIRTPDINPA	WYAGRGIRFV	50
rat.aa	1 M-ALKTWLLC	LLLLSLVLPG	ASSRAHQHSM	ETRTPDINPA	WYTGRGIRFV	50
human.aa	1 MKVLRWLLC	LLMLGLALRG	AASRTHRHSM	EIRTPDINPA	WYASRGIRFV	50
	60	70	80	90	100	
bovine.aa	51 GRFGRRRAAP	GDGPRPGPRR	VPACFRLEGG	AEPSRALPGR	LTAQLVQE*.	100
rat.aa	51 GRFGRRRATP	RDVTGLG---	QLSCLPLDGR	TKFSORG*..	.....	100
human.aa	51 GRFGRRRATL	GDVEKPGLRP	RLTCFPLEGG	AMSSQDG*..	.....	100

37/61

Fig. 36

cells;  $0.5 \times 10^7$  cells/ml[<sup>125</sup>I]-19P2-L31; 200pM (avg. 63857.3cpm)

NSB; 200nM (x 1,000)

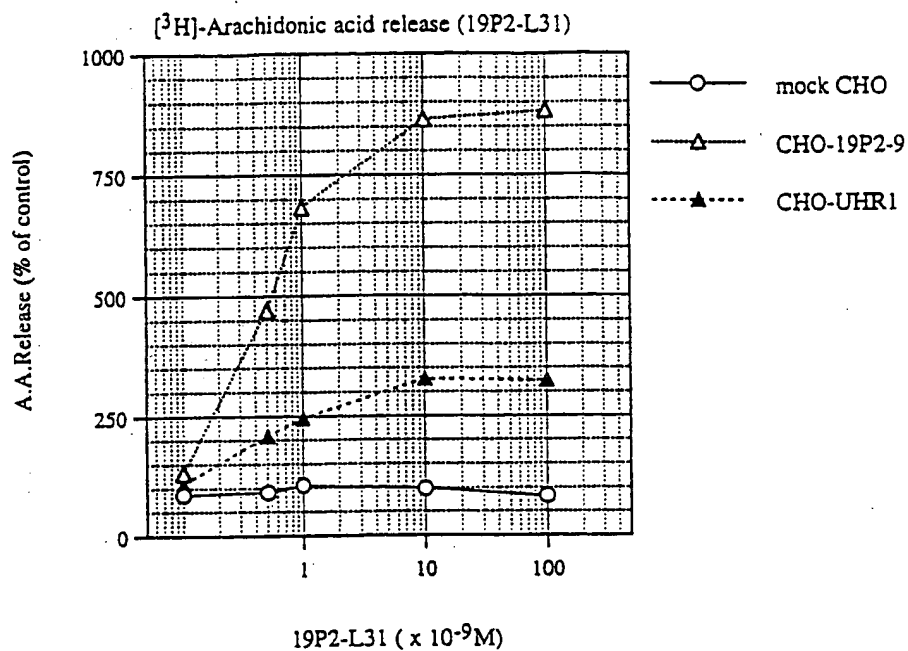
reaction; RT, 2.5hr

in HBSS + 0.05% BSA + 0.05% CHAPS

in 100  $\mu$ l

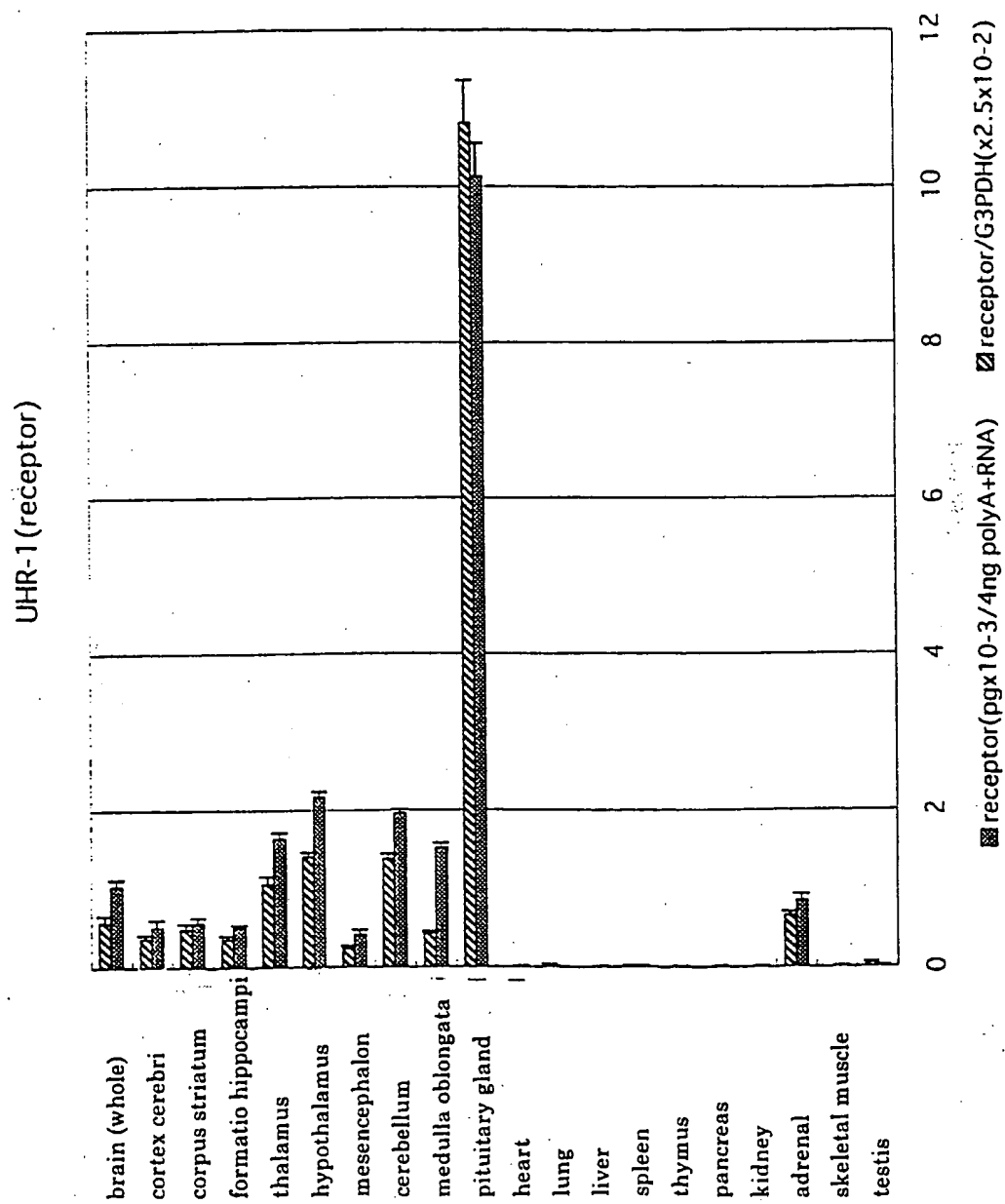
38/61

Fig. 37



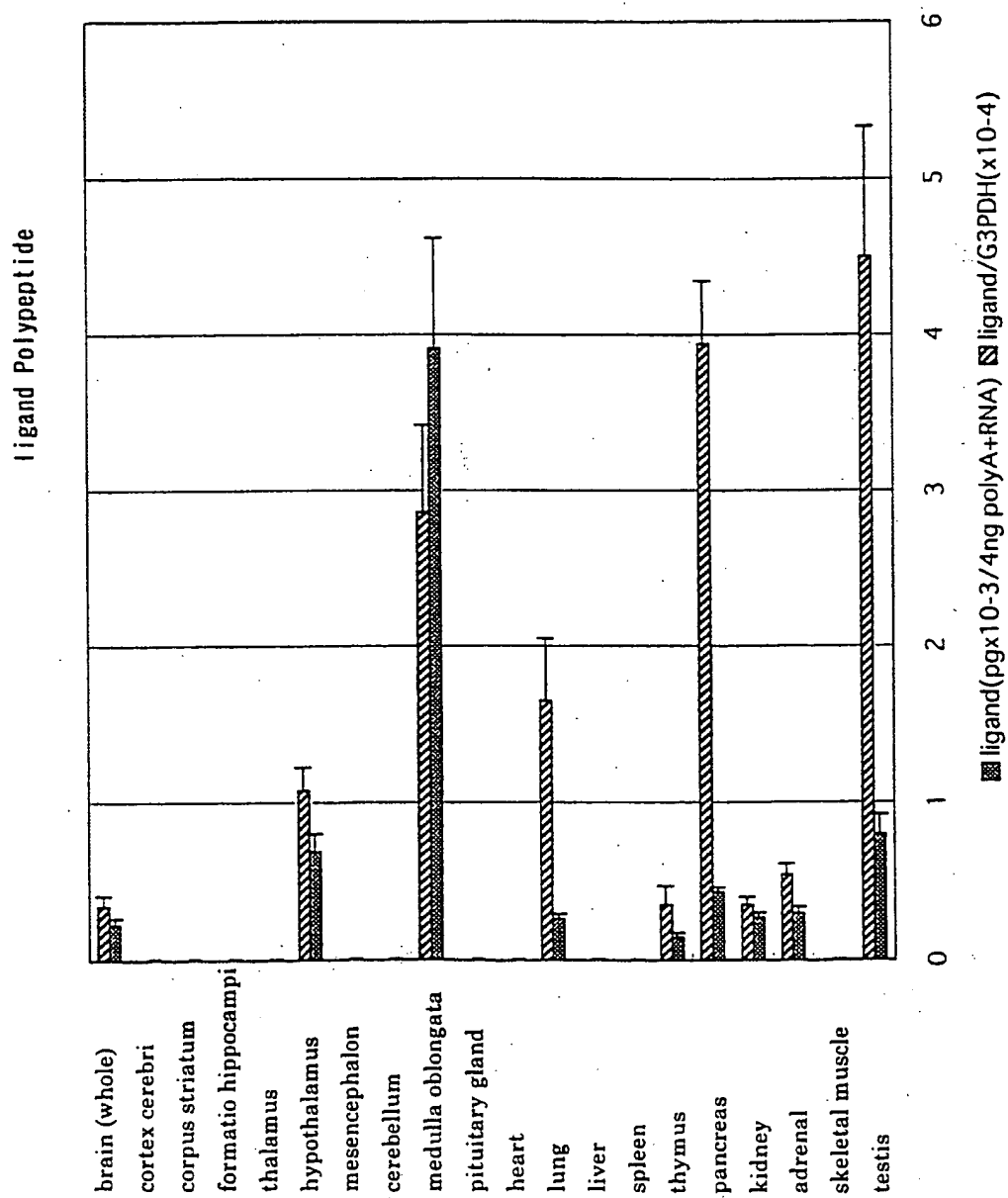
39/61

Fig. 38



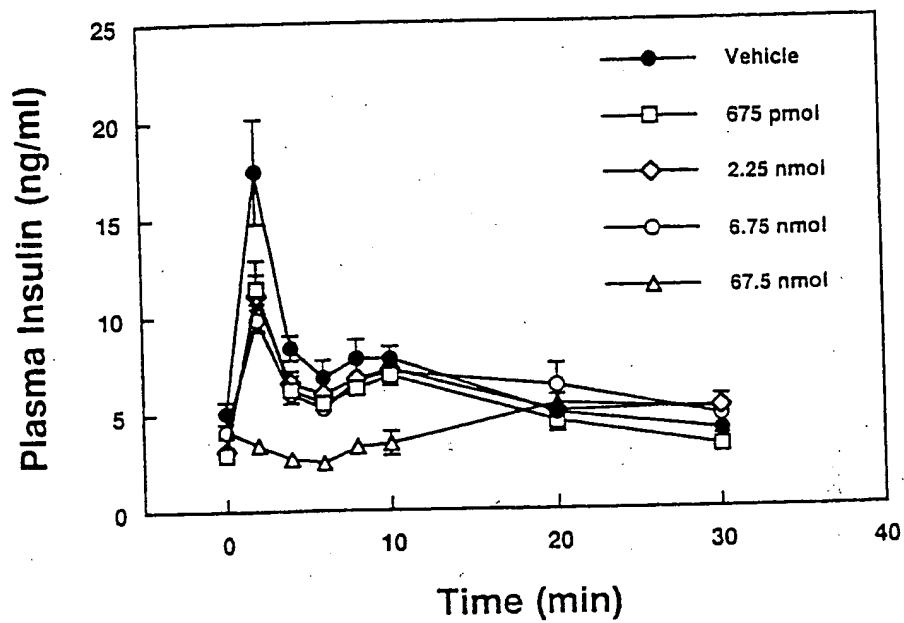
40/61

Fig. 39



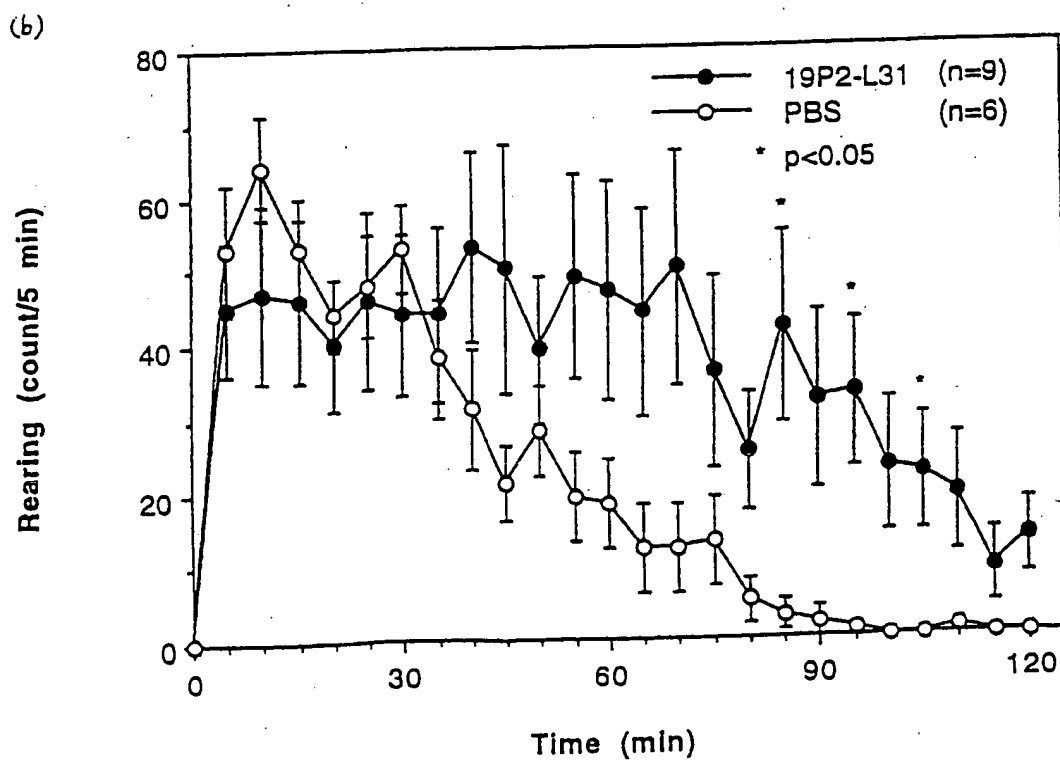
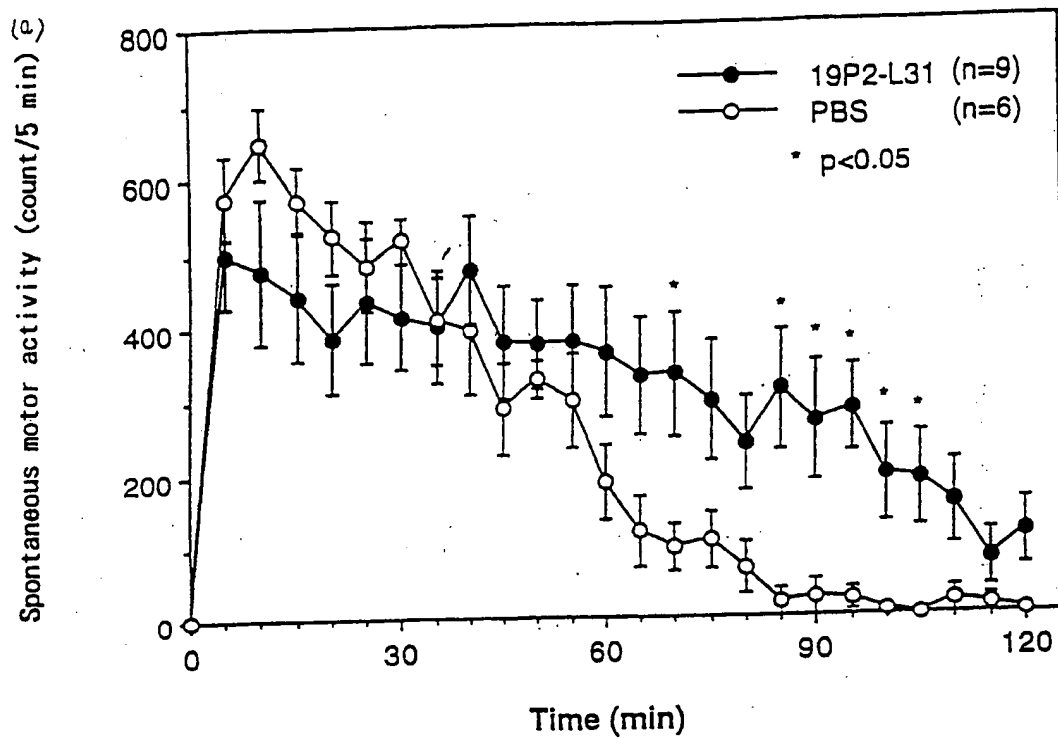
41/61

Fig. 40



42/61

Fig. 41



43/61

Fig. 42

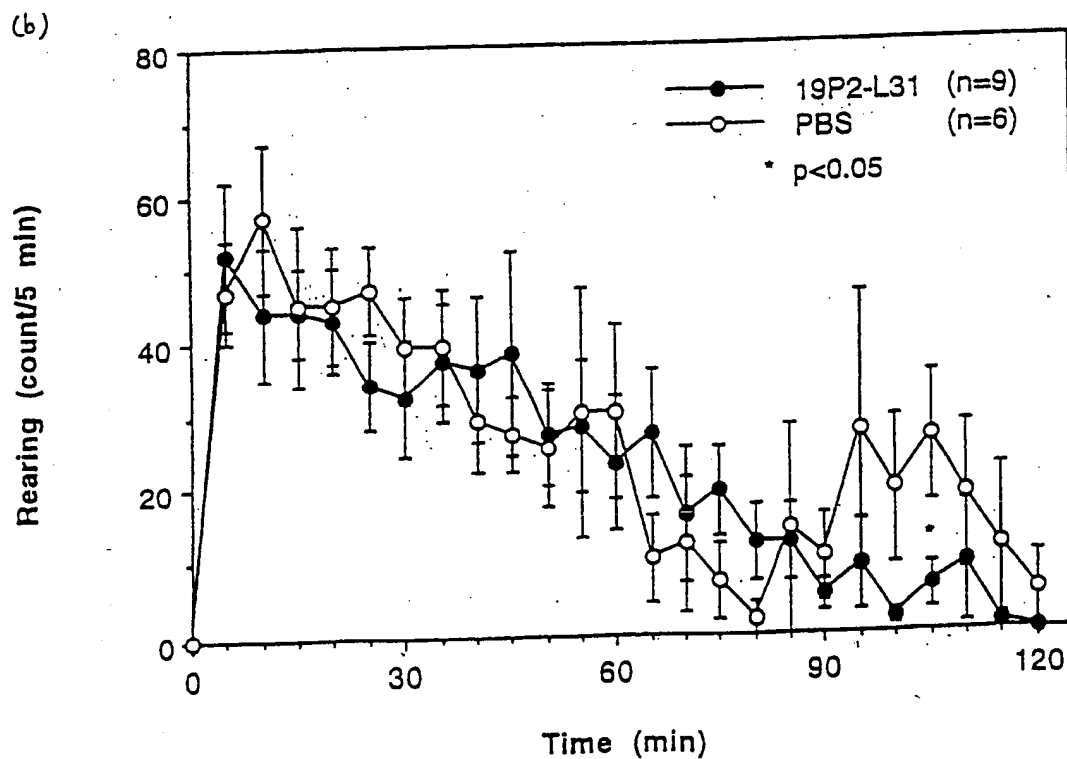
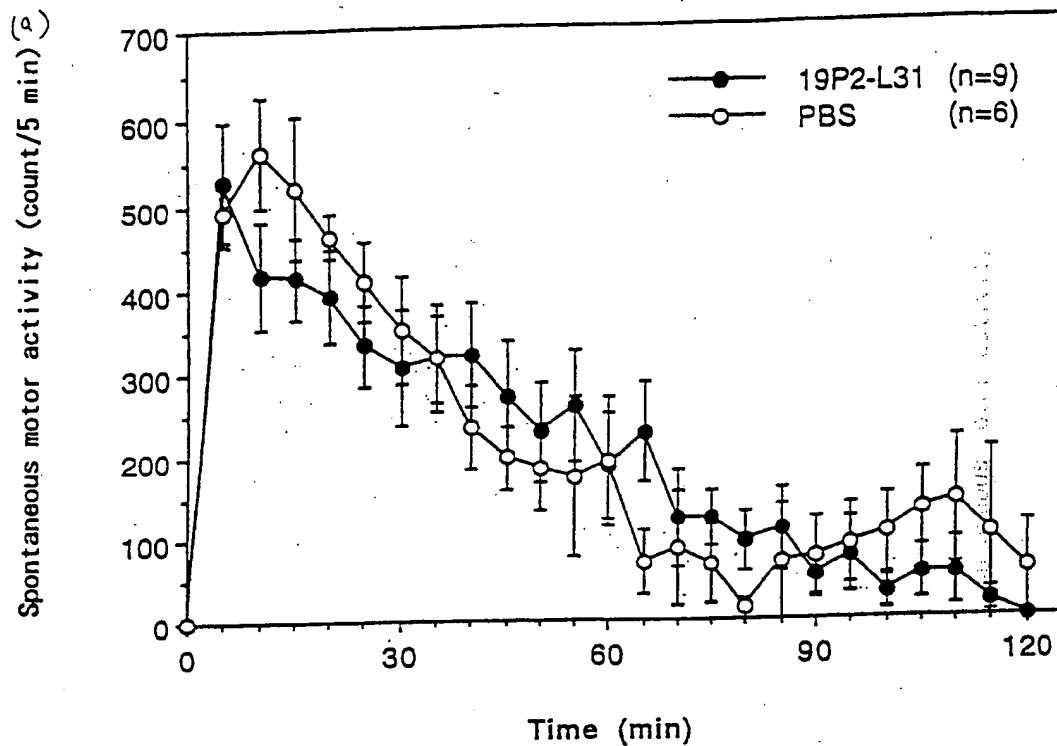
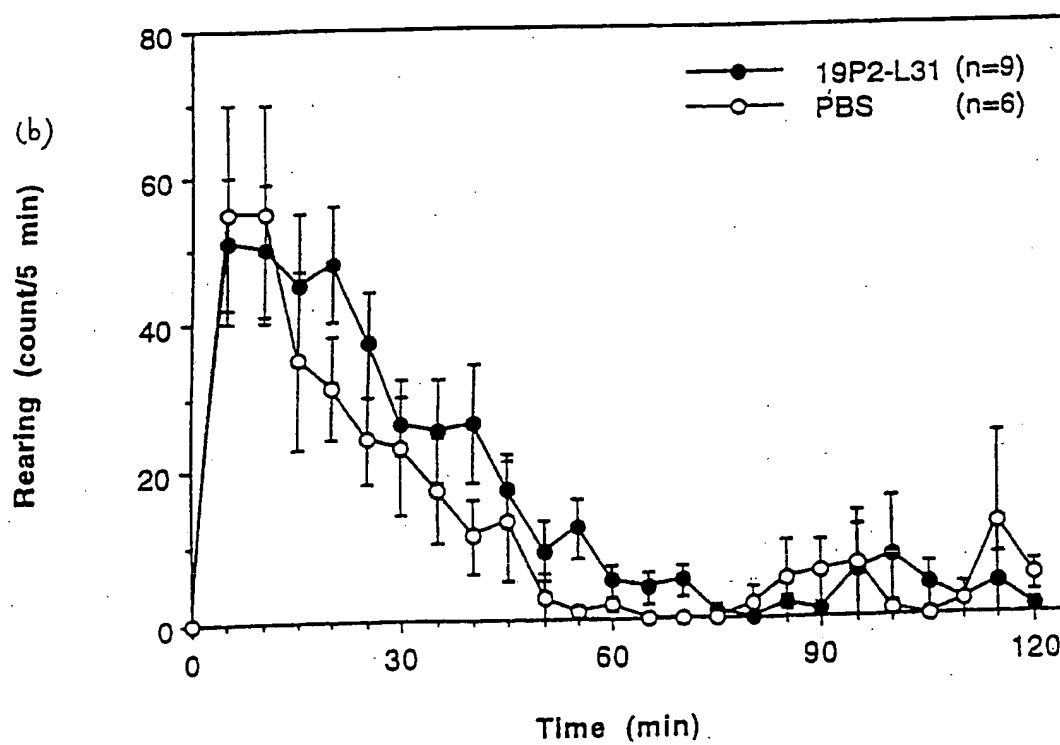
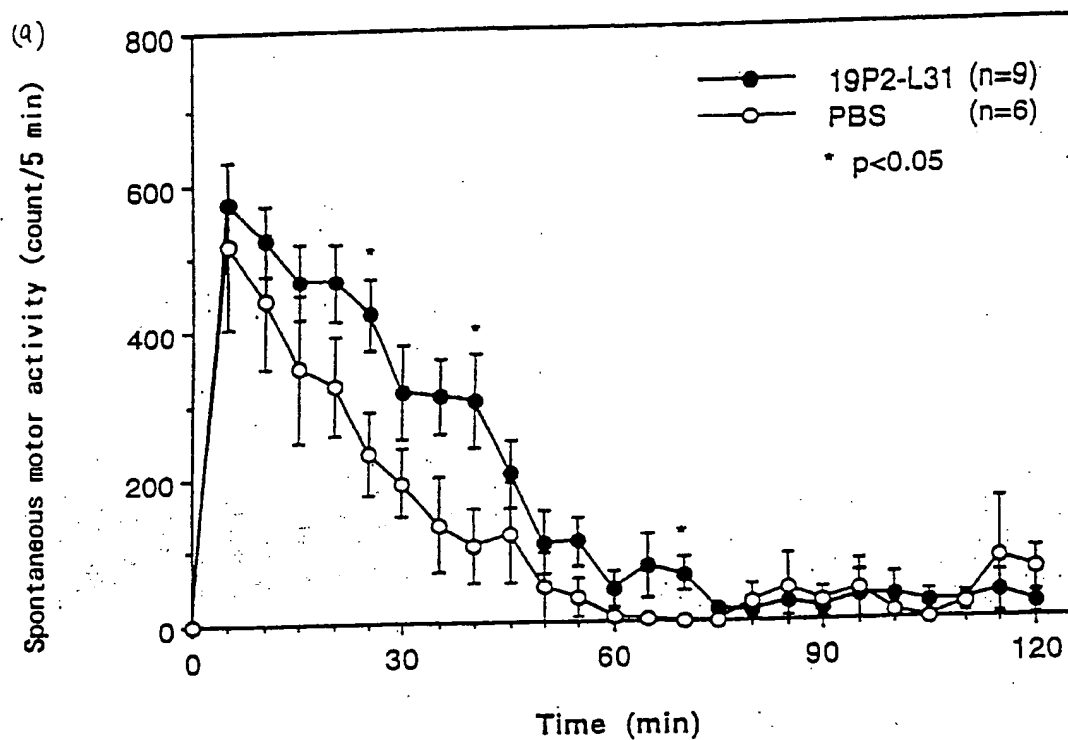


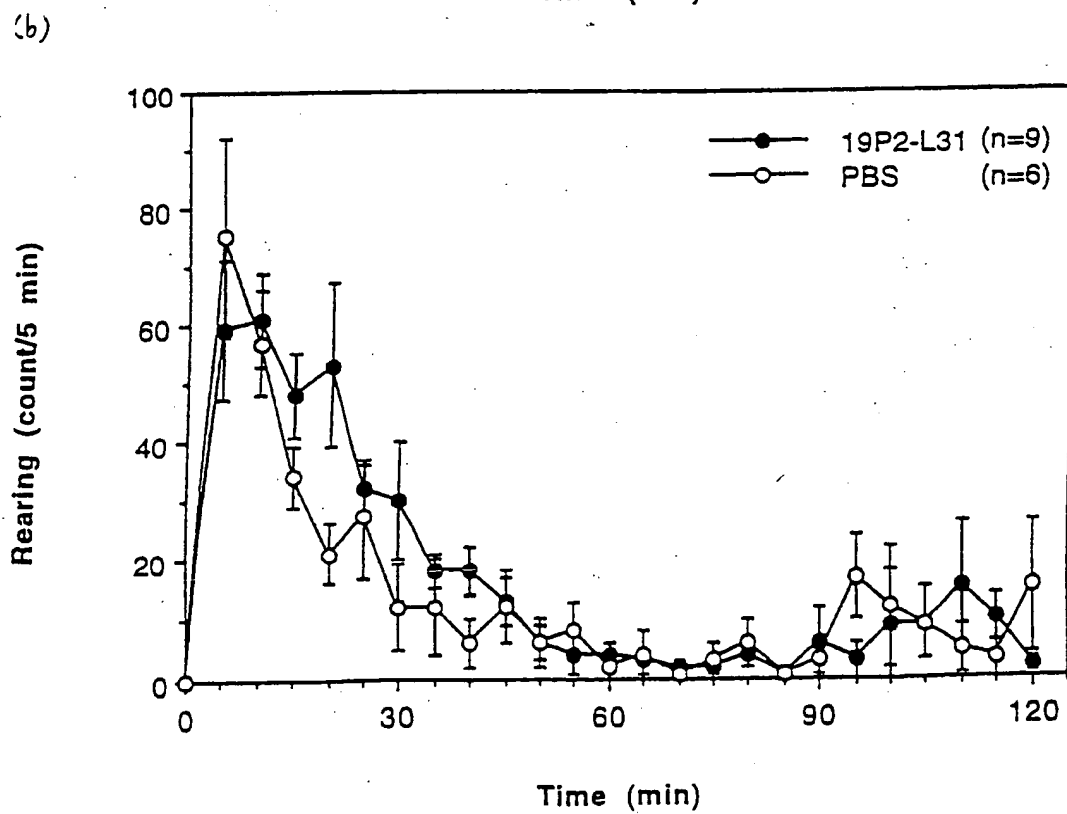
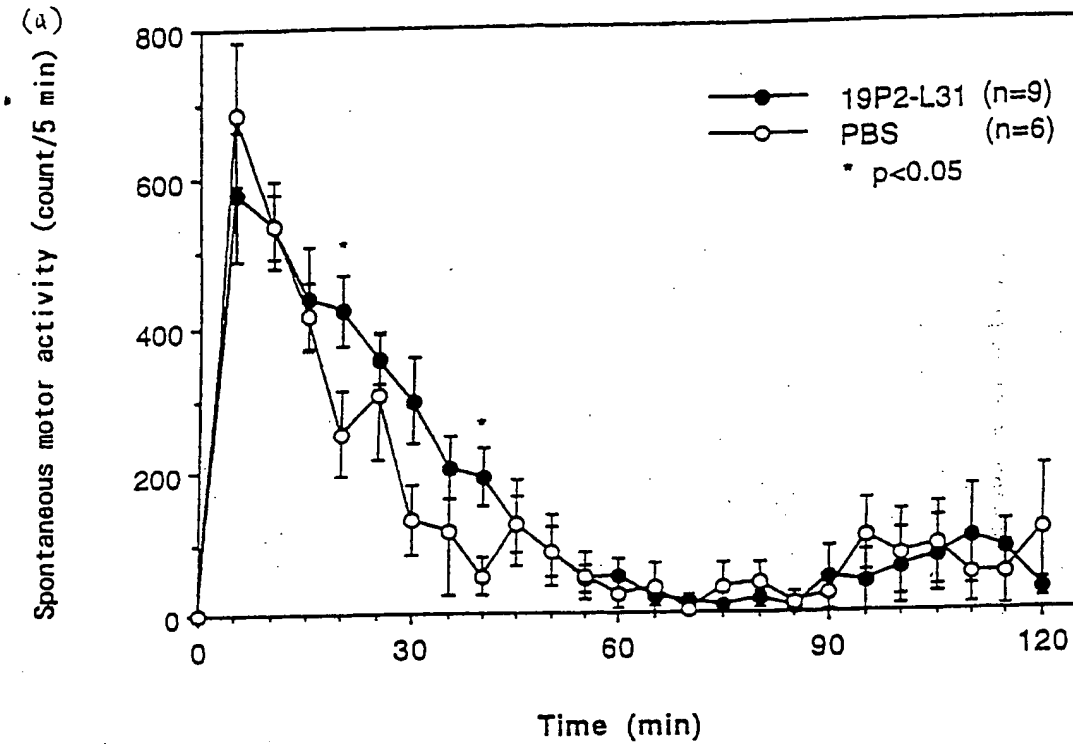


Fig. 43



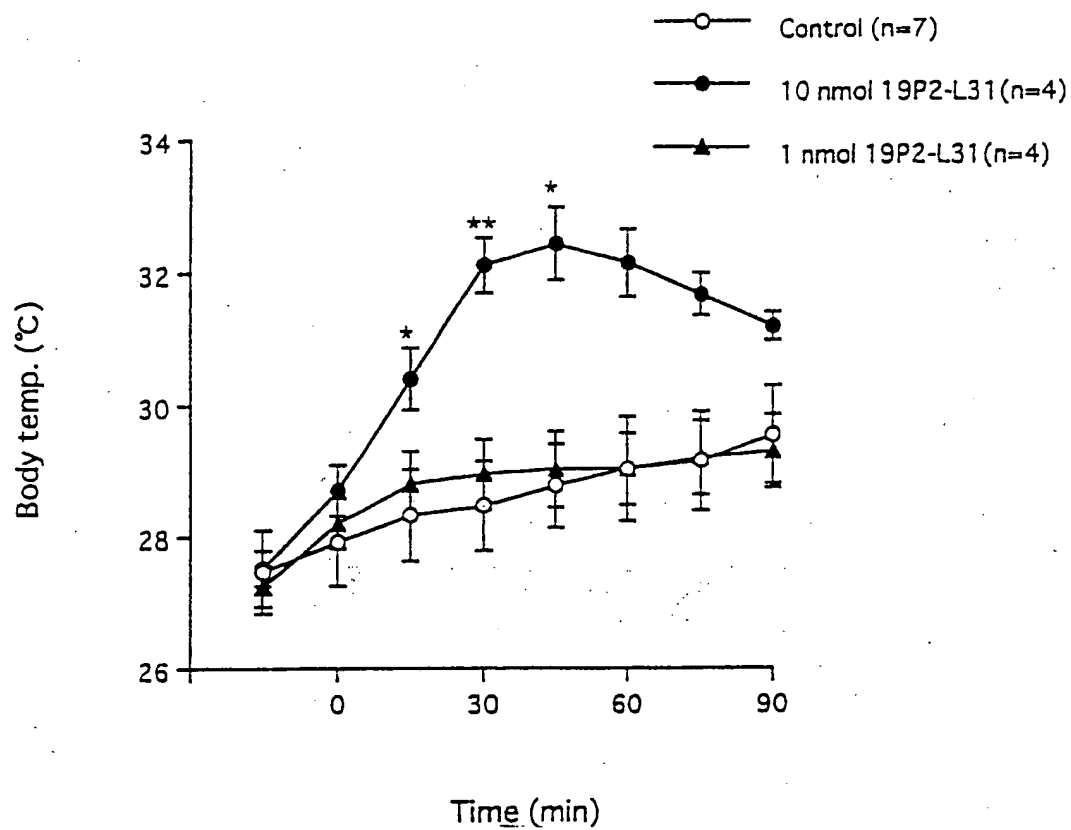
45/61

Fig. 44



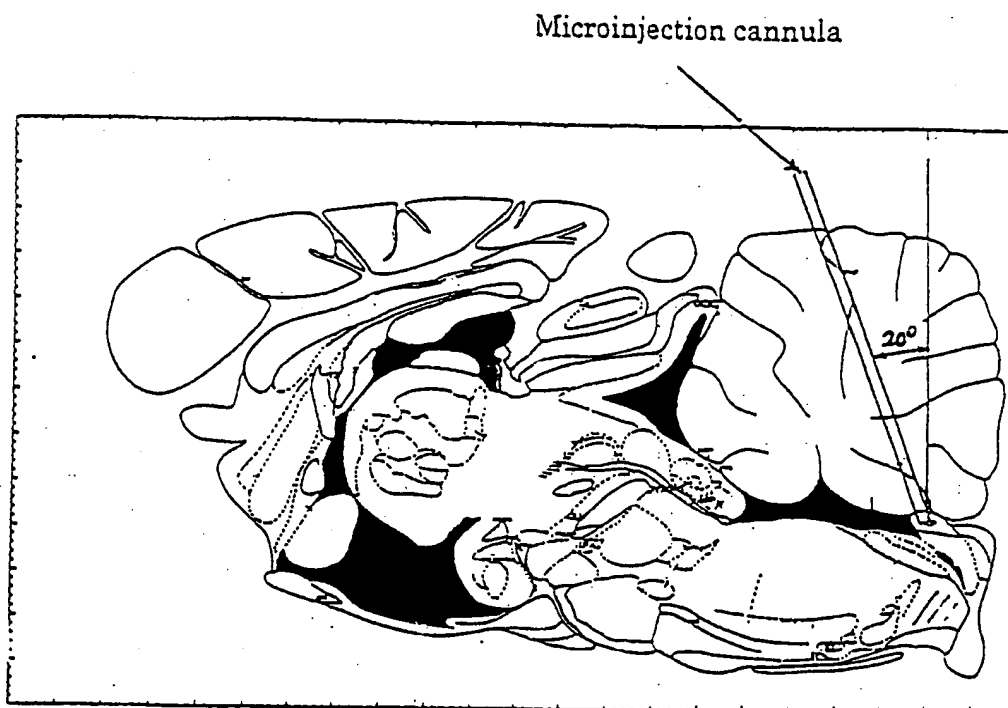
46/61

Fig. 45



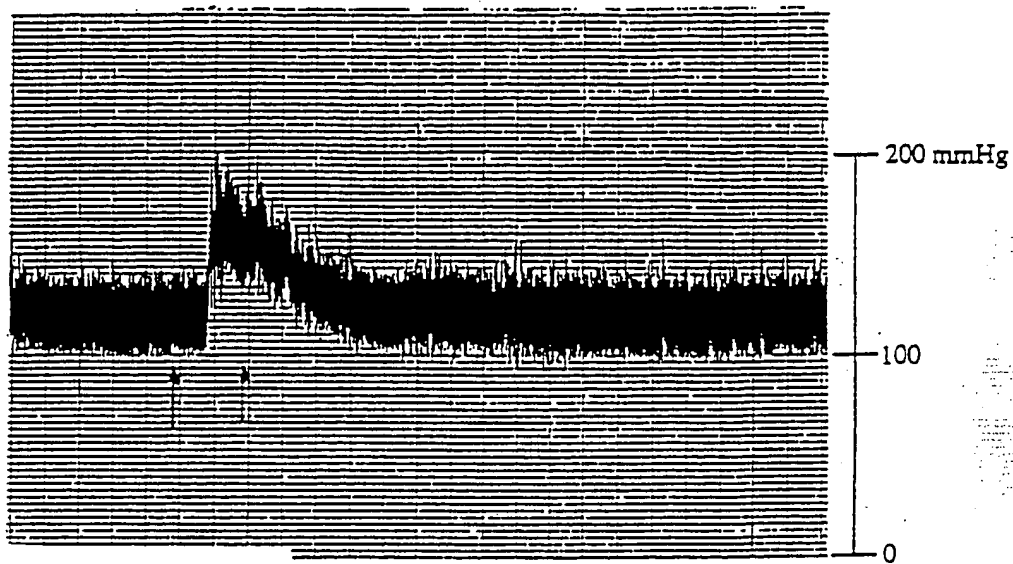
47/61

Fig. 46

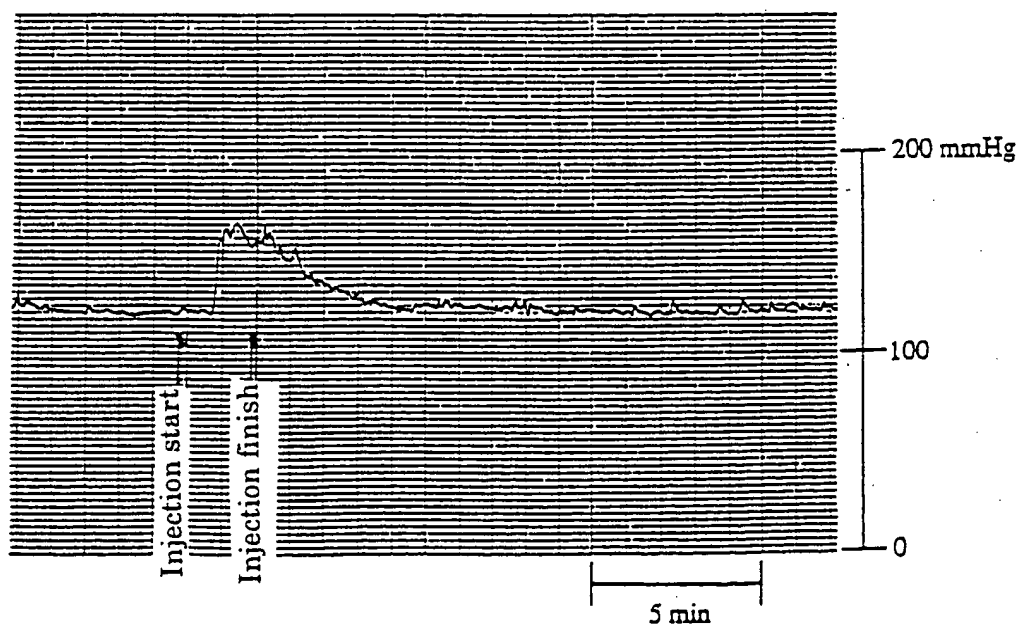


48/61

Fig. 47  
Pulse wave

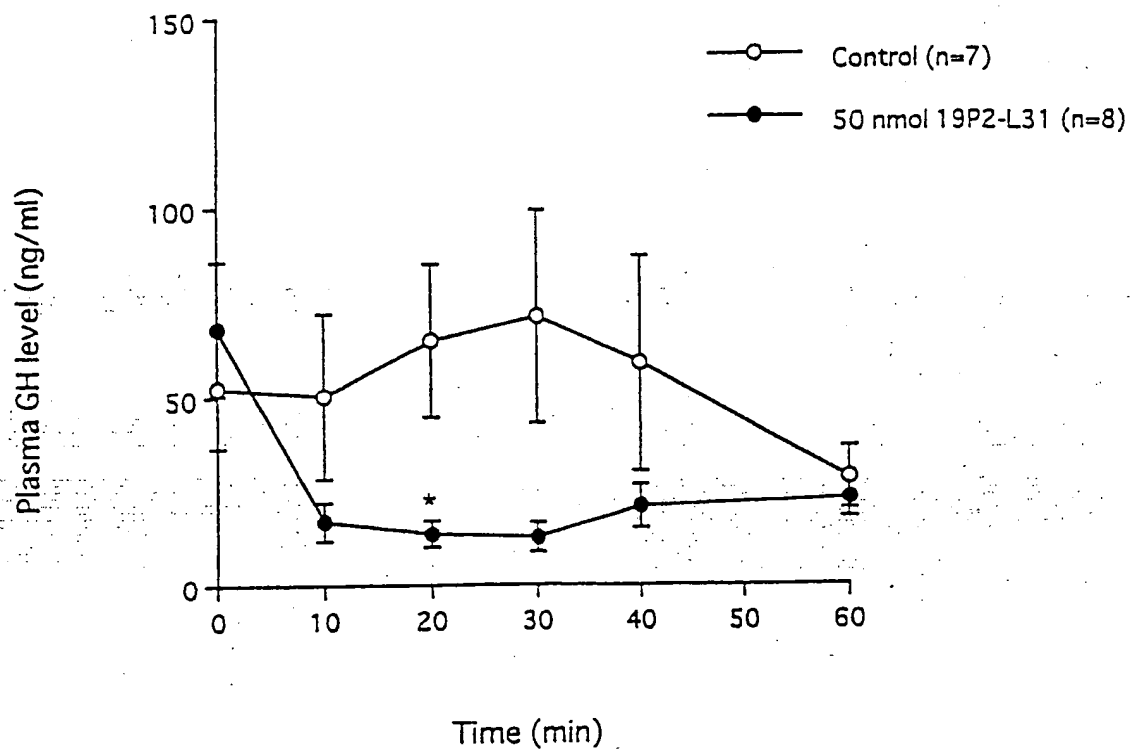


Average blood pressure

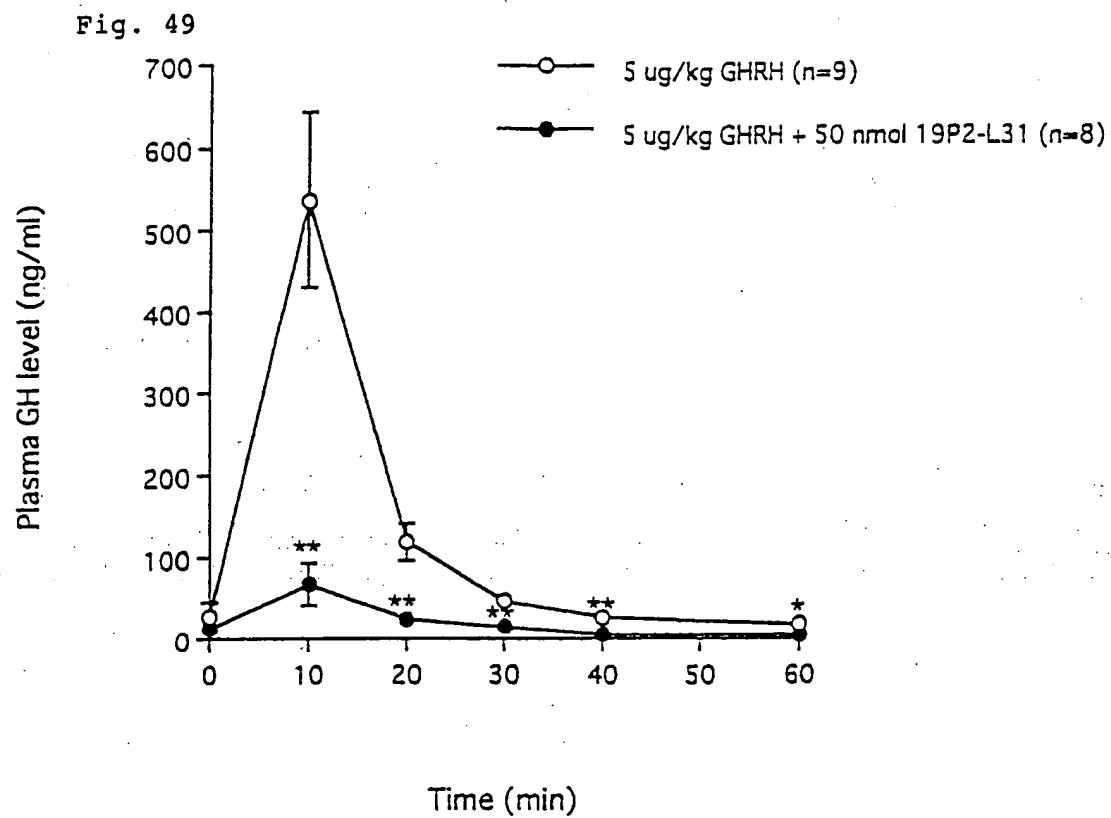


49/61

Fig. 48



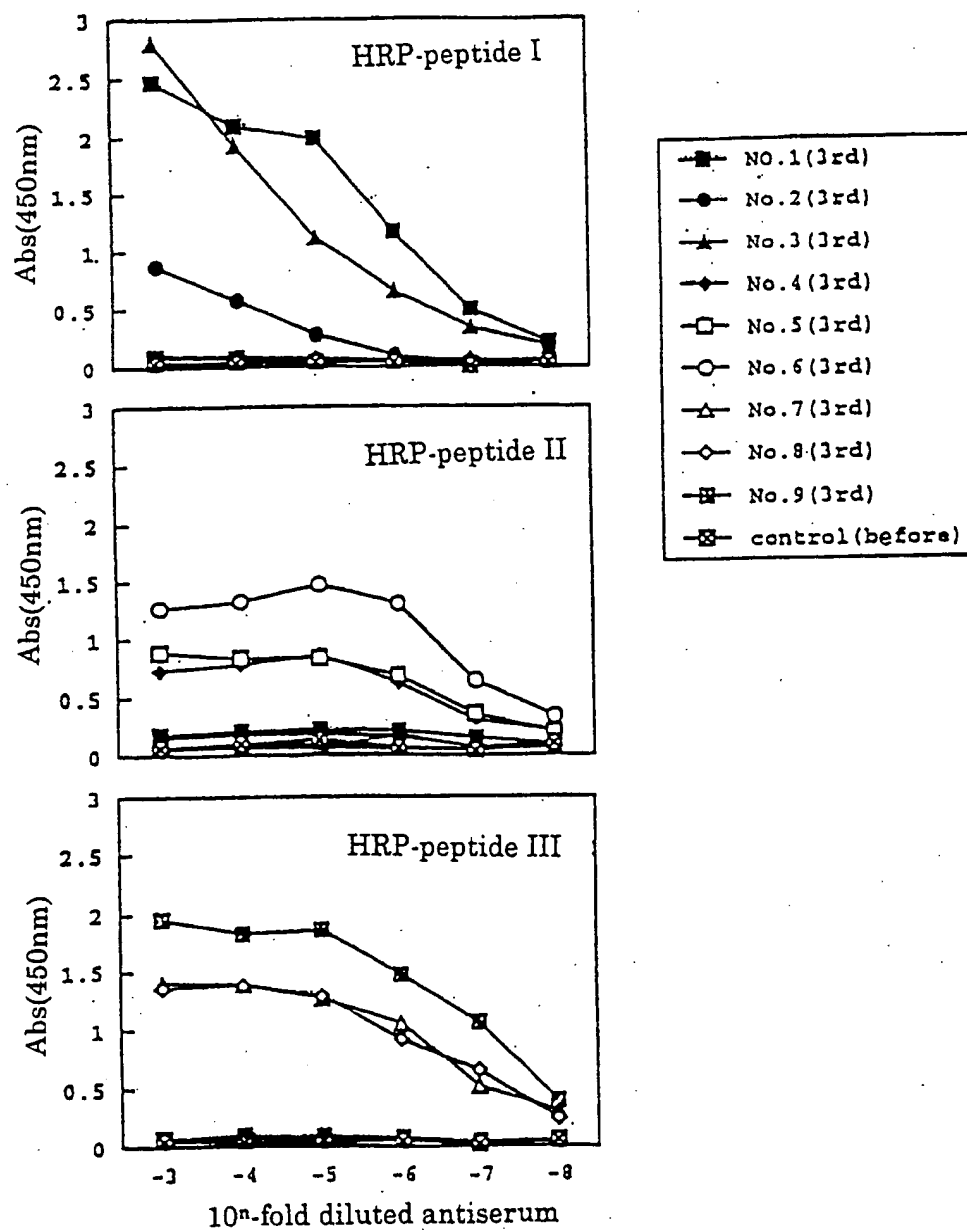
50/61



51/61

Fig. 50

Titration curve of anti-bovine 19P2 peptide I, II  
III serum using HRP-peptide I, II or III

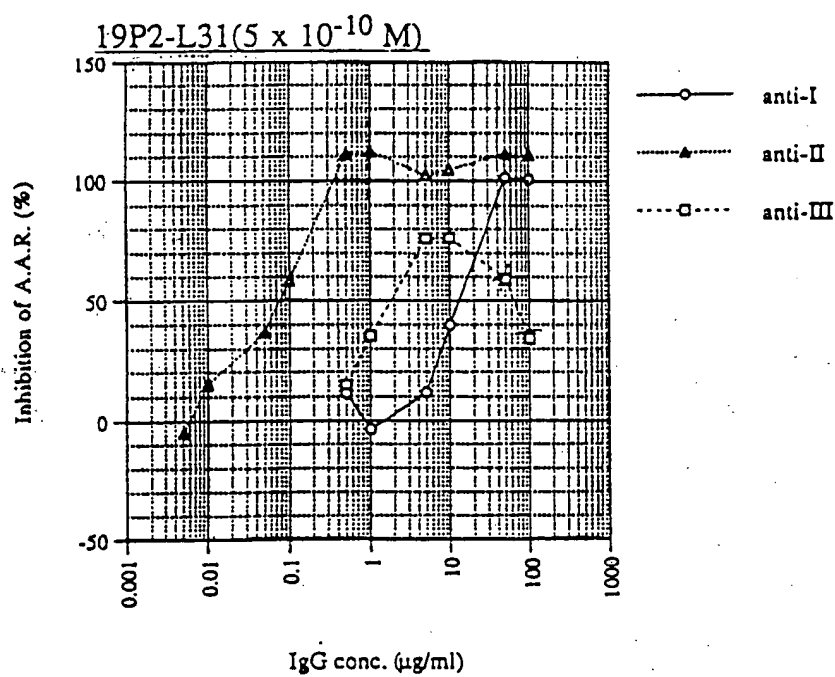




52/61

Fig. 51

Inhibition of A.A release by anti 19P2 peptide



53/61

Fig. 52

5' ATG ACC TCA GTG CCC CCT GGA ACC ACT GGG GAC CCC GAT TTG TTT TCT GGG CCG TCG CCA GGC GGC TCC ACT CCA GGC  
 Met Thr Ser Leu Pro Pro Gly Thr Thr Gly Asp Pro Asp Leu Phe Ser Gly Pro Ser Pro Ala Gly Ser Thr Pro Ala  
 81 90 99 108 117 126 135 144 153  
 AAC CAG AGT GCA GAG GCT TCA GAG AGC AAT GTG TCT GCG ACG GTT CCC AGA GCT GCA GCA GTC ACC CCG TTC CAG AGC  
 Asn Gln Ser Ala Glu Ala Ser Glu Ser Asn Val Ser Ala Thr Val Pro Arg Ala Ala Ala Val Thr Pro Phe Gln Ser  
 162 171 180 189 198 207 216 225 234  
 CTG CAA CTA GTG CAC CAG CTG AAG GGA CTG ATC GTG ATG CTG TAC AGC ATC GTG GTG GTC GTC GGT CTG GTG GGC AAC  
 Leu Gln Leu Val His Gln Leu Lys Gly Leu Ile Val Met Leu Tyr Ser Ile Val Val Val Val Gly Leu Val Gly Asn  
 243 252 261 270 279 288 297 306  
 TGC CTT CTT GTG CTG GTG ATC GCG GCG GTG CAC AAC GTG ACC AAC TTC CTC ATC GGC AAC CTG GGC TTG  
 Cys Leu Leu Val Leu Val Ile Ala Arg Val Arg Arg Leu His Asn Val Thr Asn Phe Leu Ile Gly Asn Leu Ala Leu  
 315 324 333 342 351 360 369 378 387  
 TCC GAT GTG CTC ATG TGT GGC GGC TGT GTG CTT CTC ACG CTG GGC TAC GGC TTT GAA CCT CGT GGC TCG GTG TTC GGT  
 Ser Asp Val Leu Met Cys Ala Ala Cys Val Pro Leu Thr Leu Ala Tyr Ala Phe Glu Pro Arg Gly Trp Val Phe Gly  
 396 405 414 423 432 441 450 459 468  
 GGA GGC CTG TCG CAC CTT GTT TTC TTC CTG CAG CCG GTC ACC GTC TAC GTA TCG GTG TTC ACA CTC ACC ACA ATC GCT  
 Gly Gly Leu Cys His Leu Val Phe Phe Leu Gln Pro Val Thr Val Tyr Val Ser Val Phe Thr Leu Thr Thr Ile Ala  
 477 486 495 504 513 522 531 540  
 GTG GAC CCG TAT GTG GTT CTG GTG CAC CCG CTA CGT CCG CCG ATT TCA CTG AAG CTC AGC GGC TAC GCT GTG CTG GGC  
 Val Asp Arg Tyr Val Val Leu Val His Pro Leu Arg Arg Arg Ile Ser Leu Lys Leu Ser Ala Tyr Ala Val Leu Gly  
 549 558 567 576 585 594 603 612 621  
 ATC TCG CCT CTA TCT GCA GTG CTG GCG CTG CCG GCG GCG GTG CAC ACC TAC CAT GTA GAG CTC AAG CCG CAC GAC GTG  
 Ile Trp Ala Leu Ser Ala Val Leu Ala Leu Pro Ala Ala Val His Thr Tyr His Val Glu Leu Lys Pro His Asp Val  
 630 639 648 657 666 675 684 693 702  
 CCG CTC TCG GAG GAG TTC TCG GGT TCG CAG GAG CCG CAG GGA CAG ATC TAT GGC TCG GCG CTG CTG CTG GGC ACC TAT  
 Arg Leu Cys Glu Glu Phe Trp Gly Ser Gln Glu Arg Gln Arg Gln Ile Tyr Ala Trp Gly Leu Leu Leu Gly Thr Tyr  
 711 720 729 738 747 756 765 774  
 TTG CTC CCG CTG CTG GGC ATT CTC CTG TCT TAC GTC CCG GTG TCG GTG AAG TTG CCG AAC CCG GTG GTG CCT GGC AGC  
 Leu Leu Pro Leu Leu Ala Ile Leu Leu Ser Tyr Val Arg Val Ser Val Lys Leu Arg Asn Arg Val Val Pro Gly Ser  
 783 792 801 810 819 828 837 846 855  
 GTG ACC CAG AGC CAG GCT GAC TGG GAC CCA GCG CGT CCG CGT CCG ACT TTC TCG CTG CTG GTG GTG GTG GTG GTC GTG  
 Val Thr Gln Ser Gln Ala Asp Trp Asp Arg Ala Arg Arg Arg Arg Thr Phe Cys Leu Leu Val Val Val Val Val Val  
 864 873 882 891 900 909 918 927 936  
 TTC CCG GTC TCG TCG CCG CCG CAC ATT TTC AAC CTG CTG CCG GAC CTG GAC CCG CGT GGC ATC GAC CCG TAC CCG  
 Phe Ala Val Cys Trp Leu Pro Leu His Ile Phe Asn Leu Leu Arg Asp Leu Asp Pro Arg Ala Ile Asp Pro Tyr Ala  
 945 954 963 972 981 990 999 1008  
 TTC GCG CTG GTG CAG CTC CTC TCG CAC TCG CTT GCG ATG AGC TCC GCG TCG TAC AAC CCG TTC ATC TAT CCG TCG CTG  
 Phe Gly Leu Val Gln Leu Leu Cys His Trp Leu Ala Met Ser Ser Ala Cys Tyr Asn Pro Phe Ile Tyr Ala Trp Leu  
 1017 1026 1035 1044 1053 1062 1071 1080 1089  
 CAC GAC AOC TTC CCA GAG GAG CTA CCG AAG ATG CTT CTG TCT TCG CCG CCG AAG ATC GTG CCT CAT GGC CAG AAT ATG  
 His Asp Ser Phe Arg Glu Glu Leu Arg Lys Met Leu Leu Ser Trp Pro Arg Lys Ile Val Pro His Gly Gln Asn Met  
 1098 1107 1116  
 ACC GTC AGT GTG GTC ATT TCA TCA 3'  
 Thr Val Ser Val Val Ile \*\*\*

54/61

Fig. 53

10	20	30	40	50	60
AGATCTGGCA	TCATCCAGGA	AGACGGAGCA	TGGCACCGAG	GACCTGGCTT	CTGTGCTTGC
70	80	90	100	110	120
TGCTGCTAGG	CTTAGTCCTC	CCAGGAGCTT	CCAGCCGAGC	CCACCAGCAC	TCCATGGAGA
130	140	150	160	170	180
CCCCGAGTGA	GTGCCTGGCA	TATGGAGGAC	AGCCACTGTC	ACCTCCCATC	CATATGCTTC
190	200	210	220	230	240
CCAAATGCCT	TGAGTACCCA	GCCCCCTGAAT	GGGAGGTTAG	CCATCTCCTA	AGCCAGTGGT
250	260	270	280	290	300
TTCCAACCTT	CCTAATACAG	AACITTTAAT	ACAGATCCTT	ATGTTGTGGT	GACCCCCAGC
310	320	330	340	350	360
CAGAAAATTA	TTGTGATGCT	GTTTTCATAG	TTGTAAGTTT	TGCTACTGTT	ATGGATCATA
370	380	390	400	410	420
ATGTTAATAT	CTGAAATGCA	GGATGTCGTA	TATGCGCCCT	TCCCCCCTAA	CAAAAGGGAC
430	440	450	460	470	480
ACAACCCACA	GGTTGAGAGC	CTCTGGGATC	TAAGCAAAAG	CTACCTTACC	ATGCAGTCAG
490	500	510	520	530	540
TTGGGAGATT	GGTCCTGTTA	AGATCTCCCC	AGAATGGTCC	TGTTTCCTGT	CCTCATCATT
550	560	570	580	590	600
CCCCTAACCC	ATCTTTGTGG	GGTCCCTTAA	GACTTTGGAG	GATGACAGTC	AGACAGGAAG
610	620	630	640	650	660
AGAATACTGA	TCCTGGCATA	TGTCTAAATA	AATTCCTTAA	AGCCACACCA	CTGCCCAGAT
670	680	690	700	710	720
ATGCCCAGCC	AGTGTAATCA	GGGTGGGTGC	CAACATGGCC	TGGTGCCAG	GTTTCCATCA
730	740	750	760	770	780
GCTTAGGGGC	TCCCGTGTCC	CATACGCTGC	TCTGACTCTT	TCCTTTCCAG	CCCCTGACAT
790	800	810	820	830	840
CAATCCTGCC	TGGTACACGG	GTCGTGGGAT	CAGGCCTGTG	GGCCGCTTCG	GGAGGAGGAG
850	860	870	880	890	900
GGCAGCCCTG	AGGGATGTCA	CCGGACCTGG	CCTGCGGTGC	CGGCTAAGCT	GCTTCCCACT
910	920	930	940	950	960
GGATGGAAGT	GCCAAGTTCT	CTCAGAGCTC	GAGAAGACAG	TGCTGCTGAG	TCGAC. ....

55/61

Fig. 54

AG ATC TGG CAT CAT CCA GGA AGA CGG AGC ATG GCA CCG AGG ACC TGG CTT CTG TGC  
 Met Ala Pro Arg Thr Trp Leu Leu Cys

TTG CTG CTG CTA GGC TTA GTC CTC CCA GGA GCT TCC AGC CGA GCC CAC CAG CAC  
 Leu Leu Leu Leu Gly Leu Val Leu Pro Gly Ala Ser Ser Arg Ala His Gln His

TCC ATG GAG ACC CGC A GT GAG TGC CTG GCA TAT GGA GGA CAG CCA CTG TCA CCT  
 Ser Met Glu Thr Arg

CCC ATC CAT ATG CTT CCC AAA TGC CTT GAG TAC CCA GCC CCT GAA TGG GAG GTT  
 AGC CAT CTC CTA AGC CAG TGG TTT CCA ACC TTC CTA ATA CAG AAC TTT TAA TAC  
 AGA TCC TTA TGT TGT GGT GAC CCC CAG CCA GAA AAT TAT TGT GAT GCT GTT TTC  
 ATA GTT GTA AGT TTT GCT ACT GTT ATG GAT CAT AAT GTT AAT ATC TGA AAT GCA  
 GGA TGT CTG ATA TGC GCC CTT CCC CCC AAA CAA AAG GGA CAC AAC CCA CAG GTT  
 GAG AGC CTC TGG GAT CTA AGC AAA AGC TAC CTT ACC ATG CAG TCA GTT GGG AGA  
 TTG GTC CTG TTA AGA TCT CCC CAG AAT GGT CCT GTT TCC TGT CCT CAT CAT TCC  
 CCT AAC CCA TCT TTG TGG GGT CCC TTA AGA CTT TGG AGG ATG ACA GTC AGA CAG  
 GAA GAG AAT ACT GAT CCT GGC ATA TGT CTA AAT AAA TTC CCT AAA GCC ACA CCA  
 CTG CCC AGA TAT GCC CAG CCA GTG TAA TCA GGG TGG GTG CCA ACA TGG CCT GGT  
 GCC CAG GTT TCC ATC AGC TTA GGG GCT CCC GTG TCC CAT ACG CTG CTC TGA CTC  
 TTT CCT TTC CAG CC CCT GAC ATC AAT CCT GCC TGG TAC ACG GGT CGT GGG ATC  
 Thr Pro Asp Ile Asn Pro Ala Trp Tyr Thr Gly Arg Gly Ile

AGG CCT GTG GGC CGC TTC GGG AGG AGG AGG GCA GCC CTG AGG GAT GTC ACC GGA  
 Arg Pro Val Gly Arg Phe Gly Arg Arg Arg Ala Ala Leu Arg Asp Val Thr Gly

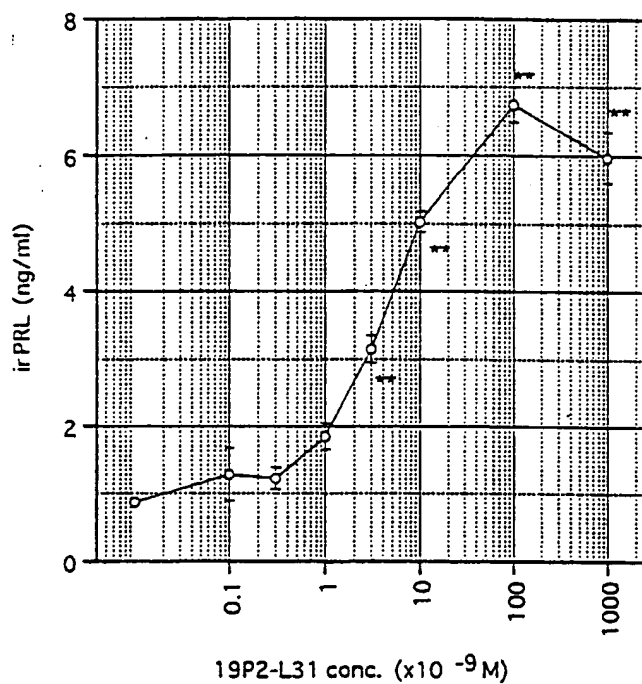
CCT GGC CTG CGG TGC CGG CTA AGC TGC TTC CCA CTG GAT GGA AGT GCC AAG TTC  
 Pro Gly Leu Arg Cys Arg Leu Ser Cys Phe Pro Leu Asp Gly Ser Ala Lys Phe

TCT CAG AGC TCG AGA AGA CAG TGC TGC TGA GTC GAC  
 Ser His Ser Ser Arg Arg Gln Cys Cys ...

56/61

Fig. 55

PRL RIA RC-4B/C P19  
Dose-Response for 30 min



Cell Culture: RC-4B/C P19

 $1 \times 10^5$ /well, for 2 Days

(12 well-plates)

(control: n=2, other points: n=4)

Wash 3 times

Pre-incubation (for 15 min)

Wash twice, Add Samples

Incubation (for 30min)

Sup. Collected, Centrifuged

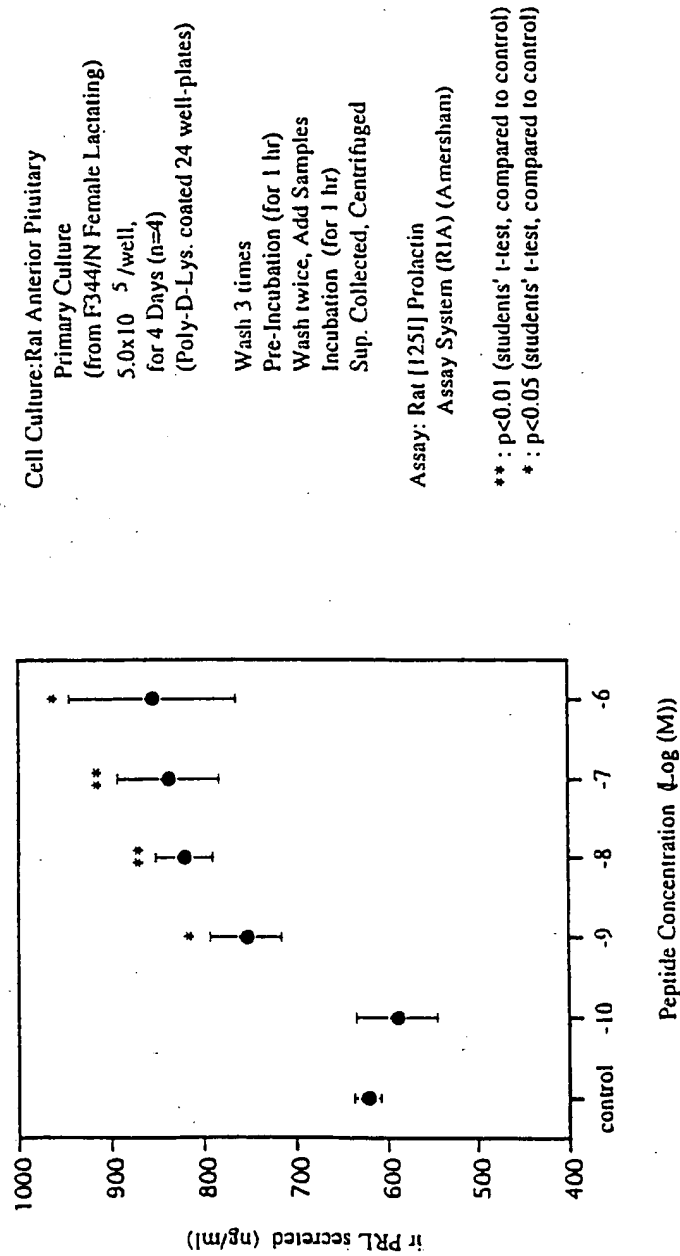
Assay: Rat [ $^{125}$ I] Prolactin

Assay System (RIA) (Amersham)

\*\*:  $p < 0.01$  (students' t-test)

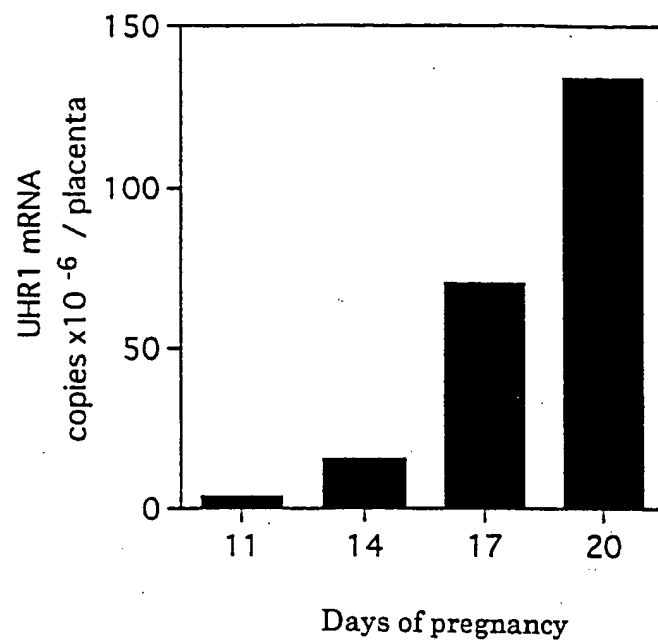
Fig. 56

Effect of bovine 19P2-L31 Peptides  
on PRL Secretion from Pituitary Cells



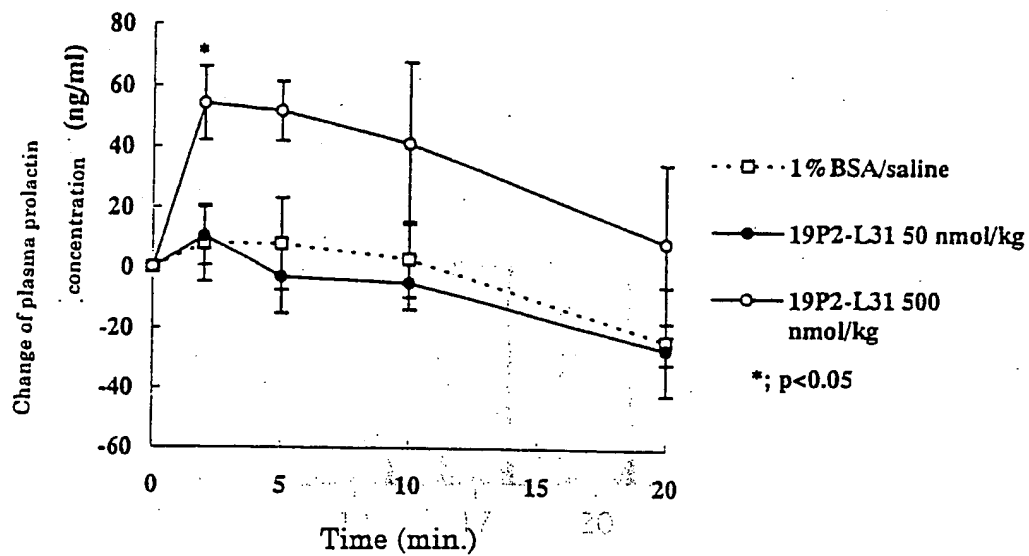
58/61

Fig. 57



59/61

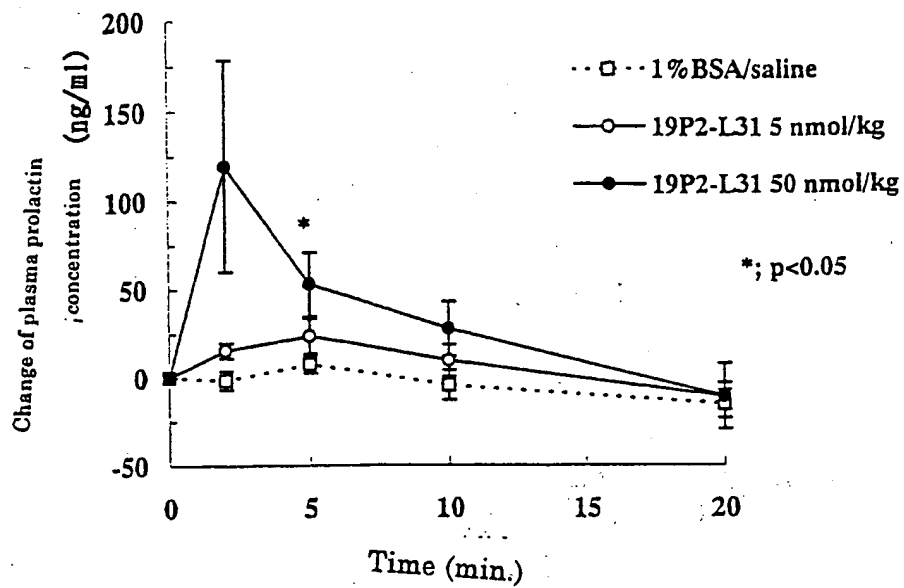
Fig. 58





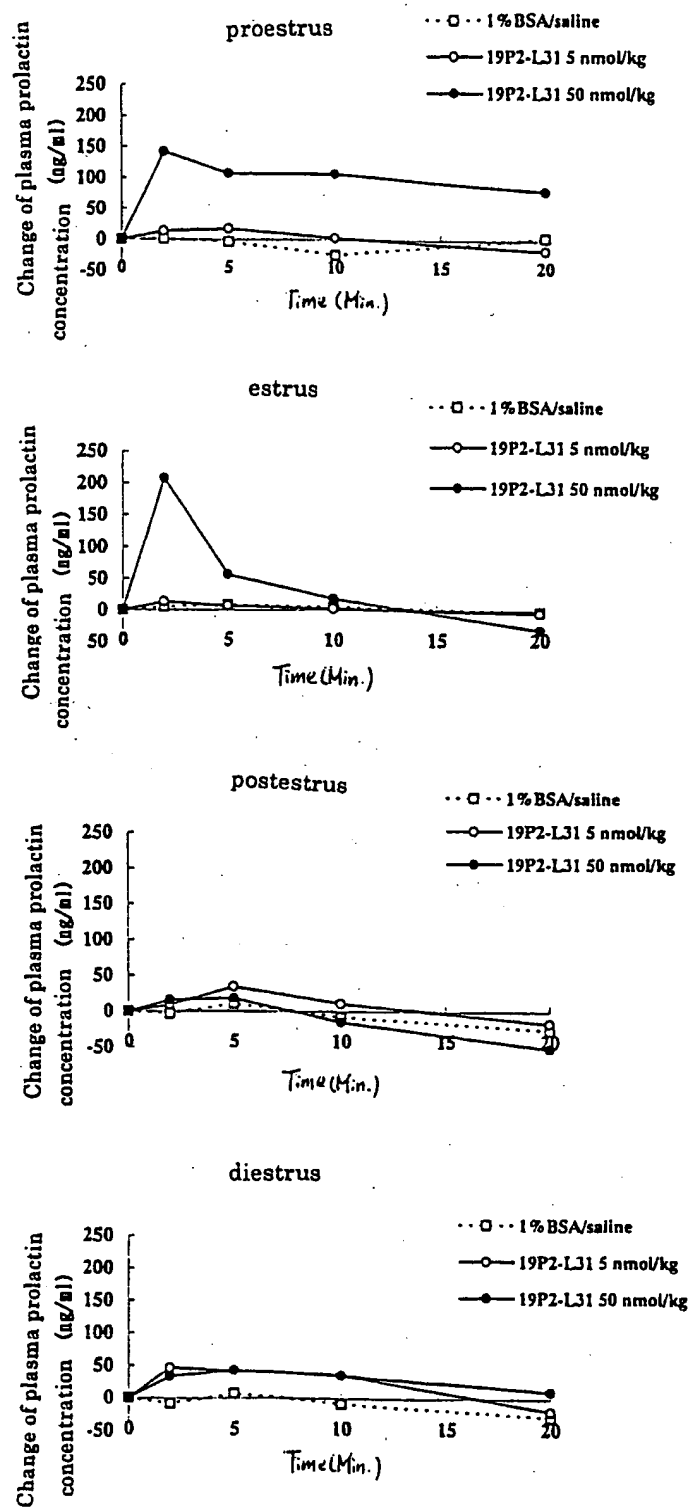
60/61

Fig. 59



61/61

Fig. 60



# INTERNATIONAL SEARCH REPORT

national Application No  
PCT/JP 98/02765

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC 6 C07K14/575 C07K14/72 A61K38/22 C07K16/26 C12N15/16  
G01N33/74

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE MEDLINE US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US ZHENG T ET AL: "Phenotypic characterization and functional correlation of alpha- MSH binding to pituitary cells." XP002084795 see abstract & AMERICAN JOURNAL OF PHYSIOLOGY, (1997 FEB) 272 (2 PT 1) E282-7. JOURNAL CODE: 3U8. ISSN: 0002-9513., United States	1,4-7, 10-14
X	WO 96 05310 A (UNIV MINNESOTA ;EL HALAWANI MOHAMED E (US)) 22 February 1996 see the whole document --- -/--	1,4-7, 10-14

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

**\* Special categories of cited documents :**

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

18 November 1998

Date of mailing of the international search report

02/12/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Groenendijk, M

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/JP 98/02765

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	XIE G -X ET AL: "EXPRESSION CLONING OF CDNA ENCODING A SEVEN-HELIX RECEPTOR FROM HUMAN PLACENTA WITH AFFINITY FOR OPIOID LIGANDS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 89, no. 9, 1 May 1992, pages 4124-4128, XP000615537 see the whole document ----	8,9,15, 16
P,X	WO 97 24436 A (FUJII RYO ;HABATA YUGO (JP); HOSOYA MASAKI (JP); KAWAMATA YUJI (JP) 10 July 1997 see the whole document ----	1-16
P,X	HINUMA S ET AL: "A prolactin -releasing peptide in the brain " NATURE, (1998 MAY 21) 393 (6682) 272-6. JOURNAL CODE: NSC. ISSN: 0028-0836., XP002084793 see the whole document ----	1-16
T	DATABASE MEDLINE US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US BUGGY J J: "Binding of alpha-melanocyte-stimulating hormone to its G-protein-coupled receptor on B-lymphocytes activates the Jak/STAT pathway." XP002084796 see the whole document & BIOCHEMICAL JOURNAL, (1998 APR 1) 331 (PT 1) 211-6. JOURNAL CODE: 9Y0. ISSN: 0264-6021., ENGLAND: United Kingdom ----	1,4-7, 10-14
A	SREEDHARAN E.A.: "Cloning and functional rxpression of a human neuroendocrine VIP receptor" BIOCHEM.BIOPHYS.RES.COMMUN., vol. 193, no. 2, 15 June 1993, pages 546-553, XP002084794 see the whole document ----	1,4-7, 10-14
A	WO 97 08317 A (CHIRON CORP ;DUHL DAVID (US)) 6 March 1997 see abstract -----	1-16

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP 98/02765

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 14 and 16 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

national Application No

PCT/JP 98/02765

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9605310	A	22-02-1996	CA	2196788 A	22-02-1996
			EP	0776367 A	04-06-1997
WO 9724436	A	10-07-1997	AU	1208497 A	28-07-1997
			EP	0870020 A	14-10-1998
			JP	10146192 A	02-06-1998
WO 9708317	A	06-03-1997	AU	7011896 A	19-03-1997